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# THE REST PERIOD OF APRICOT FLOWER BUDS AS DESCRIBED BY A REGRESSION OF TIME OF BLOOM ON TEMPERATURE[1]

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The buds of deciduous fruit trees which remain dormant when temperatures are favorable for growth are said to be in a state of rest or in the rest period. The rest influence varies in intensity, however, such that dormancy from this cause also varies and is most intense only when the rest is the deepest. Buds with lesser intensities of rest respond to favorable growing temperatures with rates of growth which vary inversely with the depth of the rest influence. It is possible, therefore, to describe the rest period by evaluating periodically the responsiveness of buds to temperatures favorable for growth. Such an evaluation is herein described for the flower buds of the Royal apricot.

The time of bloom is an end-point for measuring the rate of development of flower buds. For a given temperature favorability, the opening of the buds in the spring will depend on the amount of rest remaining after the buds have experienced the rest-breaking influence of chilling temperatures in the preceding winter. The deeper the rest remaining in the spring, other things being equal, the later will be the bloom. Bloom records of the Royal apricot, together with appropriate temperature data, were available for 24 seasons from Winters and for 20 seasons from Brentwood, California. The records were considered as 44 observations in the statistical treatment of the data, even though the same years were involved at both locations in 20 of the seasons. The dates of bloom were coded as the number of days from February 2 to full bloom inclusive.

The temperature data were obtained from weekly thermograph records. Tabulations were made of the number of hours of temperatures experienced in a series of temperature classes listed below. These data were converted to a growth unit index which expresses the favorability of temperatures for growth. Growth units were calculated by summing the products of the number of hours in the temperature classes and appropriate coefficients of temperature efficiency. This procedure weights the effectiveness of different temperatures. The coefficients of temperature efficiency which were used are based on the apparent efficiencies of temperatures in promoting the development of apricot fruits (2). Although they were derived from fruit data, they were applied here as the best approximation for weighting the effectiveness of temperatures for bud development, since no such coefficients have been derived specifically for buds. The coefficients used are as follows:

| TEMP CLASSES, ° F | COEFFICIENTS |
|---|---|
| Under 40 | 0 |
| 41– 45 | 0.002 |
| 46– 50 | 0.101 |
| 51– 60 | 0.498 |
| 61– 70 | 0.911 |
| 71– 80 | 0.989 |
| 81– 90 | 0.841 |
| 91–100 | 0.524 |
| 101–105 | 0.358 |
| Over 105 | cf text |

When hours of temperatures over 105° F were involved they were lumped with those from 101–105° F and the whole multiplied by 0.344 instead of the 0.358 used when the maximum temperatures were in the 101–105° F class. In any one day the maximum number of growth units possible is 24, which would pertain if the entire temperature experience for 24 hours were at the optimum of approximately 72° F.

## SIMPLE CORRELATIONS

The first evaluation of the effects of temperature favorability, as growth units, on time of bloom was made by calculating the simple correlation coefficients for the relation between time of bloom (number of days from February 2 to full bloom) and the number of growth units per day experienced between given dates and full bloom. In all, there are 46 dates throughout the season from which the growth units per day to full bloom were calculated. These dates include the 2nd, 9th, 16th, and 23rd of each month, beginning with March 16 and ending with the following February 16. In addition, the number of growth units per day for the entire season from full bloom to full bloom was also used. Growth units per day rather than simple growth unit summations were used in the correlations for two reasons. First, since the time of bloom is dependent on the rate of bud development, it seemed more appropriate to express the data as the rate at which favorable temperatures were experienced. Secondly, summations of data for periods between given dates and full bloom tend to reflect a bias, because the variation in time of bloom from season to season is naturally confounded with the num-

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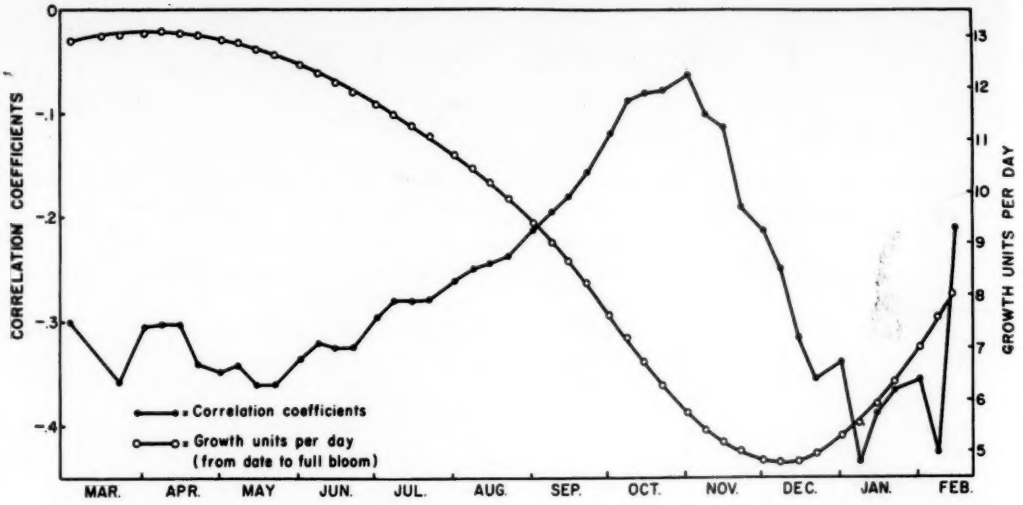
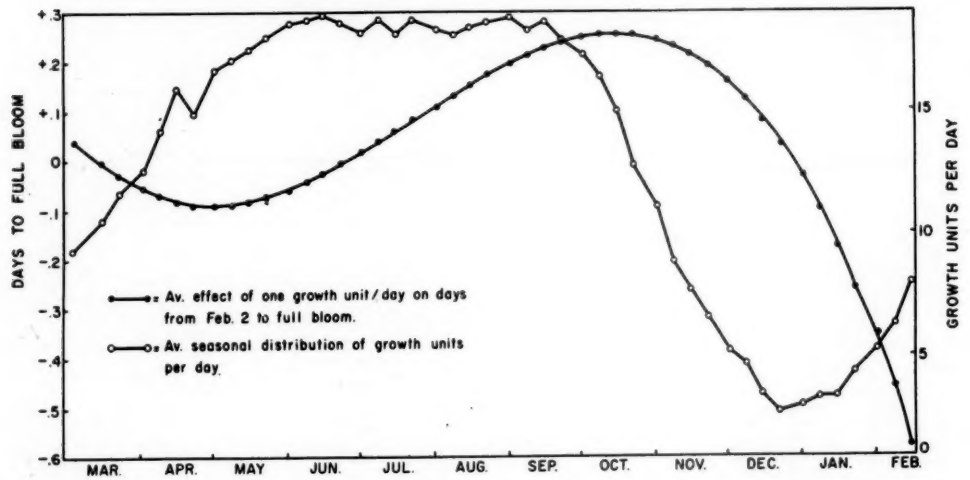
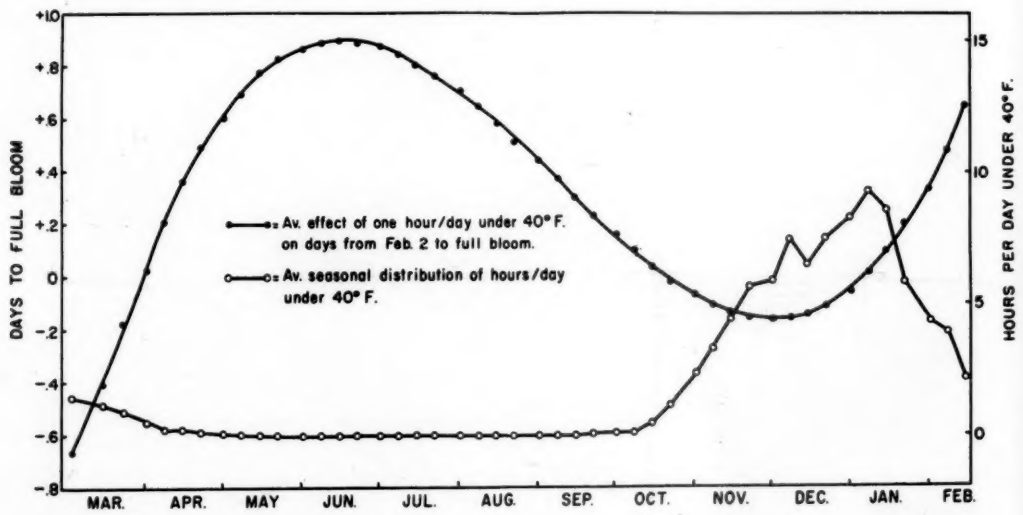

CORRELATION COEFFICIENTS
GROWTH UNITS PER DAY
= Correlation coefficients
= Growth units per day (from date to full bloom)
MAR. APR. MAY JUN. JUL. AUG. SEP. OCT. NOV. DEC. JAN. FEB.


DAYS TO FULL BLOOM
GROWTH UNITS PER DAY
= Av. effect of one growth unit/day on days from Feb. 2 to full bloom.
= Av. seasonal distribution of growth units per day.
MAR. APR. MAY JUN. JUL. AUG. SEP. OCT. NOV. DEC. JAN. FEB.


DAYS TO FULL BLOOM
HOURS PER DAY UNDER 40° F.
= Av. effect of one hour/day under 40° F. on days from Feb. 2 to full bloom.
= Av. seasonal distribution of hours/day under 40° F.
MAR. APR. MAY JUN. JUL. AUG. SEP. OCT. NOV. DEC. JAN. FEB.

ber of days in such periods; the bias can be minimized by converting the summations to a per day (rate) basis.

The correlation coefficients are plotted in figure 1. Each dot on the curve represents the correlation coefficient for the relation between time of bloom and the number of growth units per day between the date indicated by the dot and full bloom. The individual correlation coefficients are all negative, indicating an inverse relation between growth units (temperature favorability) and time of bloom. The 44-year averages of the growth units per day from successive dates to full bloom are also plotted to show the characteristic seasonal shift in the magnitude of the temperature values used in the correlations.

Considering them individually, only those coefficients with a value of $-0.3$ or larger are of statistical significance at the 5 % level and, therefore, significant as measures of the linearity of the regression of time of bloom on growth units per day from the given dates to full bloom. Considering them collectively, however, the magnitude of the coefficients varies in a distinct seasonal pattern. It is this seasonal trend in the magnitude of the coefficients which is important in this instance rather than their individual absolute values or statistical significance. The graph shows a distinct peak in late October and early November when the magnitude of the coefficients is the least; a peak formed by the coefficients of lower magnitude is possible because all the coefficients are negative and 0 is at the top of figure 1. The largest coefficients are in the January–February period.

Since correlation coefficients express the degree to which changes in one variable are associated with changes in another, the coefficients of greater magnitude in figure 1 indicate, comparatively, a closer relationship between growth units and time of bloom than is indicated by the smaller ones. But growth units are an expression of the favorability of temperatures for growth, so it follows that the larger coefficients reflect a relatively greater, and the smaller coefficients comparatively a lesser, responsiveness of buds to favorable temperatures. The October–November peak of smaller coefficients in figure 1 is, therefore, the period when the buds are least responsive to growing temperatures. This must be the period of deepest rest. The curve in figure 1 suggests that on the average the rest influence gradually deepens from late June, becoming deepest in early November. Thereafter, the buds emerge rapidly from the rest and become quite responsive to growing temperatures by mid-January.

## Regression Analysis

Growth Unit Data: The foregoing description of the rest period based on temperatures between given dates and full bloom merely evaluates past experience and does not offer much promise in the way of indexing seasonal progress of buds in a new year. Therefore, the prediction technique described by Houseman (3) was applied in calculating the regression of time of bloom on the seasonal distribution of the temperature experience. Houseman's procedure is an application of a method developed by R. A. Fisher to examine the influence of meteorological factors on crop yield. It gives a regression curve which shows the effect on yield of a unit change in a given climatic factor at any time during the growing season. It is used here to describe the effect on time of bloom of a unit change in growth units per day (temperature favorability) at 46 different times during the season from full bloom to full bloom. The ordinate of a point on the regression curve corresponding to any particular time represents the mean effect, in days to full bloom, of one growth unit per day at that time.

To develop the regression curve, the growth units per day were calculated for each of the 46 periods throughout the season in each of the 44 seasons of observation. The first period was from full bloom to March 15 and the last from February 16 to full bloom. Between these two, growth units per day were calculated for the periods of the 2nd to the 8th, the 9th to the 15th, the 16th to the 22nd, and the 23rd to the 1st, of each month. Houseman's technique was then applied to these data to formulate a regression curve; a third degree polynomial gave the best fit to the data. The regression accounted for 41 % of the total sums of squares and was highly significant ($F = 6.81$ for 4 and 39 d.f.).

The regression curve is plotted in figure 2. The 44-year averages of growth units per day for each of the 46 periods are also plotted to show the seasonal characteristics of the distribution of the temperature favorability on which the regression is based. This plot of growth units per day shows the average temperature favorability for each of the 46 short intervals into which the season was divided; it therefore differs from that in figure 1 which shows the average number of growth units per day between given dates and full bloom. Each point on the regression curve indicates for its respective period the average effect, in tenths of a day, of one growth unit per day on the time of full bloom. These coefficients may be used to predict the time of bloom at any one of the 46 periods

Fig. 1 (*top*). Plot of correlation coefficients for the relation between the time of bloom and the temperature favorability (growth units) from given dates to full bloom, together with a plot of the averages of the growth unit data used in the calculations.

Fig. 2 (*center*). Regression curve (plotted on dots) expressing the relation between the time of bloom and the seasonal distribution of growing temperatures (expressed as growth units/day), together with the averages (plotted on circles) of the growth unit data used in the calculations.

Fig. 3 (*bottom*). Regression curve (plotted on the dots) expressing the relation between the time of bloom and the seasonal distribution of chilling temperatures (hrs/day under 40° F), together with the averages (plotted on circles) of the temperature data used in the calculations.

HOURS PER DAY UNDER 40° F.

during the season. To do this, the actual algebraic deviation from average of the growth units per day for each interval up to the given period is multiplied by the appropriate coefficient. The sum of such products added to the mean number of days to full bloom gives the estimated time of bloom as of the end of the given interval.

Actually, however, the bloom prediction value of the regression is not its most interesting or practical feature in this instance. Rather, it is the relative effectiveness of the favorable temperatures at different times during the season which should be considered. The similarity in conformation between the regression curve in figure 2 and the plot of the correlation coefficients in figure 1 points to similarities in interpretation with respect to the rest period. Favorable temperatures during October and November add days to the average time of bloom. Similar temperatures experienced nearer to bloom subtract days from the average time. Comparatively, therefore, it is indicated that the buds are least responsive to favorable temperatures in the fall coincident with the time of deepest rest. As the rest is broken during the winter, the buds rapidly become responsive to favorable growing temperatures.

Chilling Temperatures: Since temperatures below 40° F are known to have a rest-breaking influence and, therefore, an effect on bloom, a regression of time of bloom on the number of hours per day under 40° F was also calculated. The regression curve is plotted in figure 3, along with the average number of hours per day below 40° F for each of the 46 periods during the season. The regression was highly significant ($F = 4.08$ for 4 and 39 d.f.), accounting for 30 % of the total sums of squares.

The curve indicates that temperatures below 40° F in mid-summer (if actually experienced) would lengthen the time to bloom. Likewise, as the time of bloom approaches, chilling temperatures tend to delay bloom. In general, the curve bears out the recognized effectiveness of chilling from late November through January in breaking the rest and thereby shortening the time to bloom. Surprisingly, a relatively greater effectiveness per hour under 40° F in shortening the time to bloom is indicated during March and early April than in the November to January period. At least, if the hours under 40° F were to be used in predicting time of bloom the regression coefficients for March in particular would yield a greater reduction in the time to bloom per hour under 40° F than those for December.

Combined Analysis: The same regression technique also permits a simultaneous consideration of both the chilling effect and temperature favorability (growth unit) effect, resulting in an evaluation of each factor independently of the other. The results of such a combined analysis are presented in figure 4 which shows (A) the regression curves for growth units per day with and without the effects of the hours under 40° F removed, and (B) the regression curves for the hours per day under 40° F with and without the effects of the growth units removed.

In figure 4 A the curve plotted on the dots is the same as that in figure 2 and represents the effects on time of bloom of growth units per day without consideration of the effects of the hours under 40° F. The line in figure 4 A plotted on the circles is derived from the combined analysis and represents the effects on time of bloom of a growth unit per day independent of the effect of the hours under 40° F. Thus, the values of the first curve express the relationship of growth units to time of bloom as it is modified by chilling temperatures, whereas those of the second curve express the relationship free of that modifying influence. The differences between the curves indicate, therefore, the extent to which chilling hours (under 40° F) modify the effectiveness of growing temperatures in shortening the time to full bloom.

In figure 4 B the curve plotted on the dots is the same as that in figure 3 and represents the effects on time of bloom of an hour per day under 40° F without consideration of the effects of growing temperatures. The curve plotted on the circles is derived from the combined analysis and represents the effects on time of bloom of an hour per day under 40° F independent of the influence of growth units. The differences between the two curves indicate the extent to which growing temperatures modify the effectiveness of hours under 40° F in shortening the time to full bloom.

Relative Effects: The absolute values of the curves in either pair in figure 4 are not fully satisfactory for comparing one curve with another in the interpretation of rest period effects. To begin with, the differences in algebraic sign are awkward to handle in trying to evaluate differences between the curves. More important, however, is the fact that the absolute values do not express directly the relative values of the week to week effectiveness of growth units or of chilling hours in advancing time of bloom. The relative values are important because the interpretation is based on the premise that the degree of rest influence is related to the seasonal change in effectiveness of one growth unit or of one hour under 40° F per day on time of bloom. By transposing the absolute values to a relative basis, interpretation is facilitated.

The curves in figure 4 were converted to a relative basis by recalculating the values of each curve as percentages of the range in values for the individual curves. This made it necessary to assign values of 0 and 100 % to the extreme values on each curve. This was done on the basis of the relative effectiveness of the growth units or of the chilling hours in shortening the time to full bloom. Positive values indicate a lengthening of the time to bloom and so, comparatively, a lower effectiveness than is represented by negative values. Therefore, on each curve the positive value of greatest magnitude represents 0 % effectiveness and the negative value of greatest magnitude 100 % effectiveness. The points on a curve between

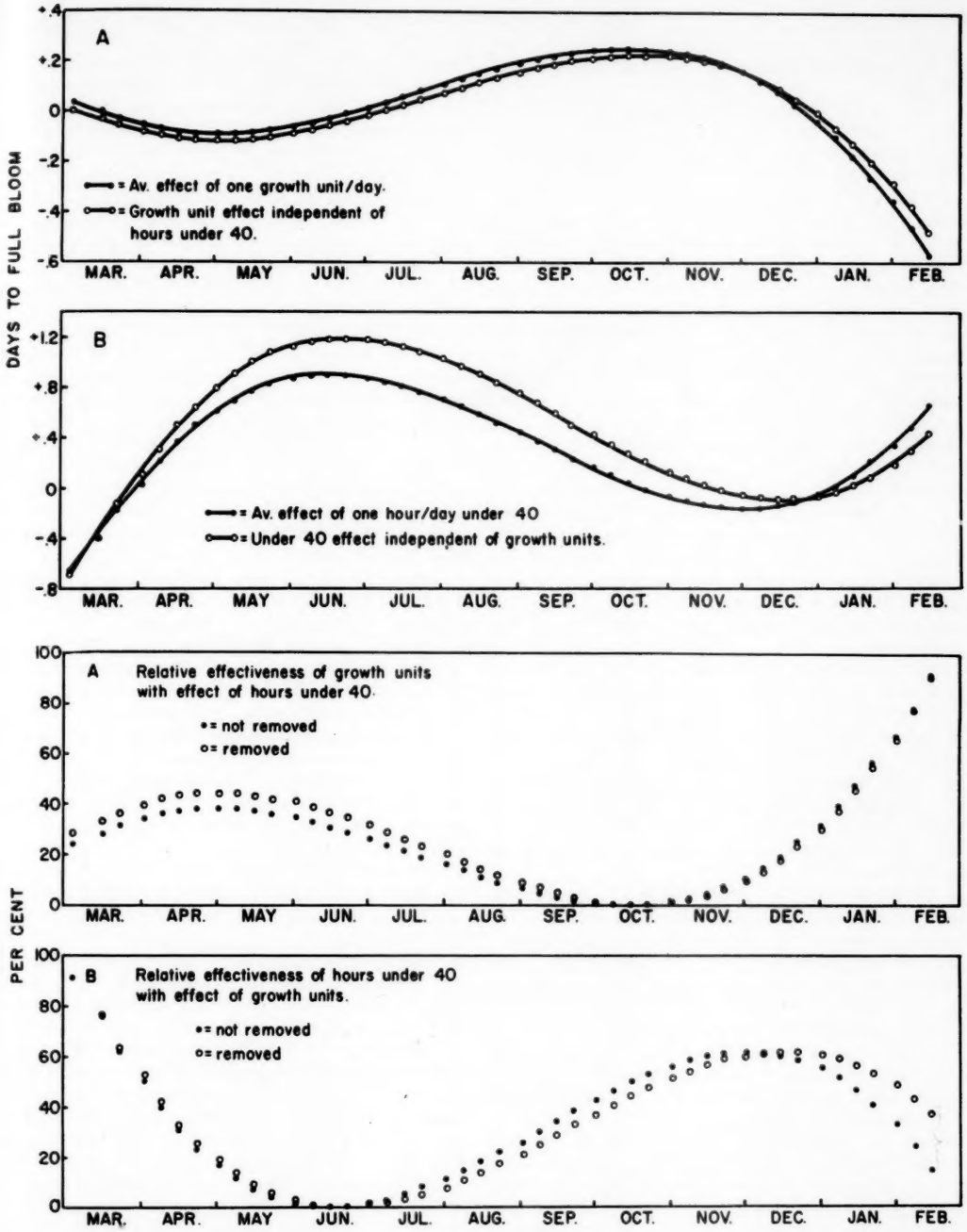



FIG. 4 (*top*). A. The regression curves expressing the relation between time of bloom and the distribution of temperatures favorable for growth (growth units) with and without the effects of chilling temperatures removed. B. The regression curves expressing the relation between time of bloom and the distribution of chilling temperatures (hrs under 40° F) with and without the effects of growing temperatures removed.

FIG. 5 (*bottom*). A. Relative effectiveness of favorable growing temperatures (growth units) with and without the effects of chilling temperatures removed. B. Relative effectiveness of chilling temperatures (hrs under 40° F) with and without the effects of growing temperatures removed.

these extremes have relative values between 0 and 100 %.

The curves in figure 5 are the result of converting those in figure 4 to a relative basis. In figure 5 A, the curve plotted as dots represents the relative effectiveness of growth units, from period to period, *without* the effects of hours under 40° F removed; it was derived from the curve plotted on the dots in figure 4 A. Similarly, the curve in figure 5 A plotted on the circles represents the relative effectiveness of growth units, from period to period, *with* the effects of hours under 40° F removed; it was derived from the curve plotted on the circles in figure 4 A. The terminology "with (or without) the effects of the hours under 40° F removed" as applied in figure 5 A follows from the method of calculating the regression curves of figure 4 A. The curve plotted on the dots was derived in an analysis which included only the growth unit data, so that the portion of the variance attributable to the effects of hours under 40° F was not accounted for (was not removed). The curve plotted on the circles, however, was derived in an analysis which included both the growth unit and the chilling data, so that portions of the variance could be assigned to each; that is, the effects of hours under 40° F were separated (removed) from the growth unit part of the regression.

In like manner, the curve plotted as the dots in figure 5 B represents the relative effectiveness of hours under 40° F, from period to period, *without* the effects of growth units removed; it was derived from the curve plotted on the dots in figure 4 B. The curve in figure 5 B plotted on the circles represents the relative effectiveness of hours under 40° F, from period to period, *with* the effects of growth units removed; it was derived from the curve plotted on the circles in figure 4 B.

Chilling Efficiency: In figure 5 A, the growth unit curve with the effects of hours under 40° F removed represents the relative seasonal effectiveness (in advancing time of bloom) of temperatures favorable for growth independent of the influence of chilling. On the other hand, the curve without the effects of hours under 40° F removed represents the seasonal effectiveness of growing temperatures as modified by the effects of chilling. Therefore, the differences between corresponding points on the two curves afford a measure of the influence of hours under 40° F on growth unit effectiveness. For example, in the first period, the relative effectiveness of growth units without the effects of chilling removed is 24.3 %, as compared to a value of 28.5 % with the effects of chilling removed. The independent effectiveness of the growth units is greater; it was decreased 4.2 % under the influence of hours under 40° F. In general, although the magnitude of such differences is not large, they do show that the effectiveness of growing temperatures in hastening time of bloom is reduced by chilling temperatures from early spring until October with the greatest reduction occurring in the week of May 9 to 15. For that week the difference between the curves is −6.3 (the value of the curve plotted on the dots minus that of the curve plotted on the circles, figure 5 A). From the end of October to full bloom chilling temperatures increase the relative effectiveness of growing temperatures, the maximum increase occurring from December 16 to January 15. For that time, the difference between the curves is 1.6.

The influence of hours under 40° F on the effectiveness of temperatures favorable for bud growth is two-fold. First, the occurrence of hours under 40° F in any interval of time limits the effectiveness of growing temperatures by reducing the possible number of hours in the favorable range by an amount equal to the number of hours under 40° F experienced. At the same time it causes a greater proportion of the growing temperatures to be experienced at considerably below optimum efficiency. Secondly, hours under 40° F increase the effectiveness of temperatures favorable for growth by breaking the rest period of the buds and making the buds more responsive to favorable growing temperatures. The periods during the season in which chilling causes the sharpest reduction in the relative effectiveness of growing temperatures are times when the first effect of hours under 40° F predominates and the rest-breaking influence is at a minimum. Conversely, the periods when chilling temperatures are associated with the greatest improvement in the effectiveness of growing temperatures are times when the second effect—the rest-breaking influence—is predominant.

These two effects of hours under 40° F account for the differences between the growth unit curves of figure 5 A. Consequently, the differences between comparable points on the two curves will serve as a basis for estimating (a) the degree to which the relative effectiveness of growth units is modified by the influence of hours under 40° F, and (b) the rest-breaking influence of chilling as expressed through the relative improvement it induces in the effectiveness of growing temperatures. When differences are taken between comparable points on the two curves, the values for the different periods vary in algebraic sign. As in figure 4, the variable signs are awkward to handle. Furthermore, the interest is mainly on a comparative or relative evaluation of one period with another. Therefore, the differences between the curves of figure 5 A were transposed to percentages of the seasonal range in differences. The zero value corresponds to that in the May 9 to 15 period when the greatest reduction (−6.3) in the effectiveness of growing temperatures occurs. The 100 % value corresponds to the maximum positive difference of 1.6 which occurs during the period of December 16 through January 15. Other differences between the curves in figure 5 A were adjusted to values between 0 and 100 % and are shown graphically by the line plotted on the dots in figure 6 A. The term "relative efficiency" is applied to the values on this curve. This is done to distinguish this evaluation of growth unit effectiveness from that in figure 5 A; the term "efficiency" is applicable since the evaluation weights the growth unit influence

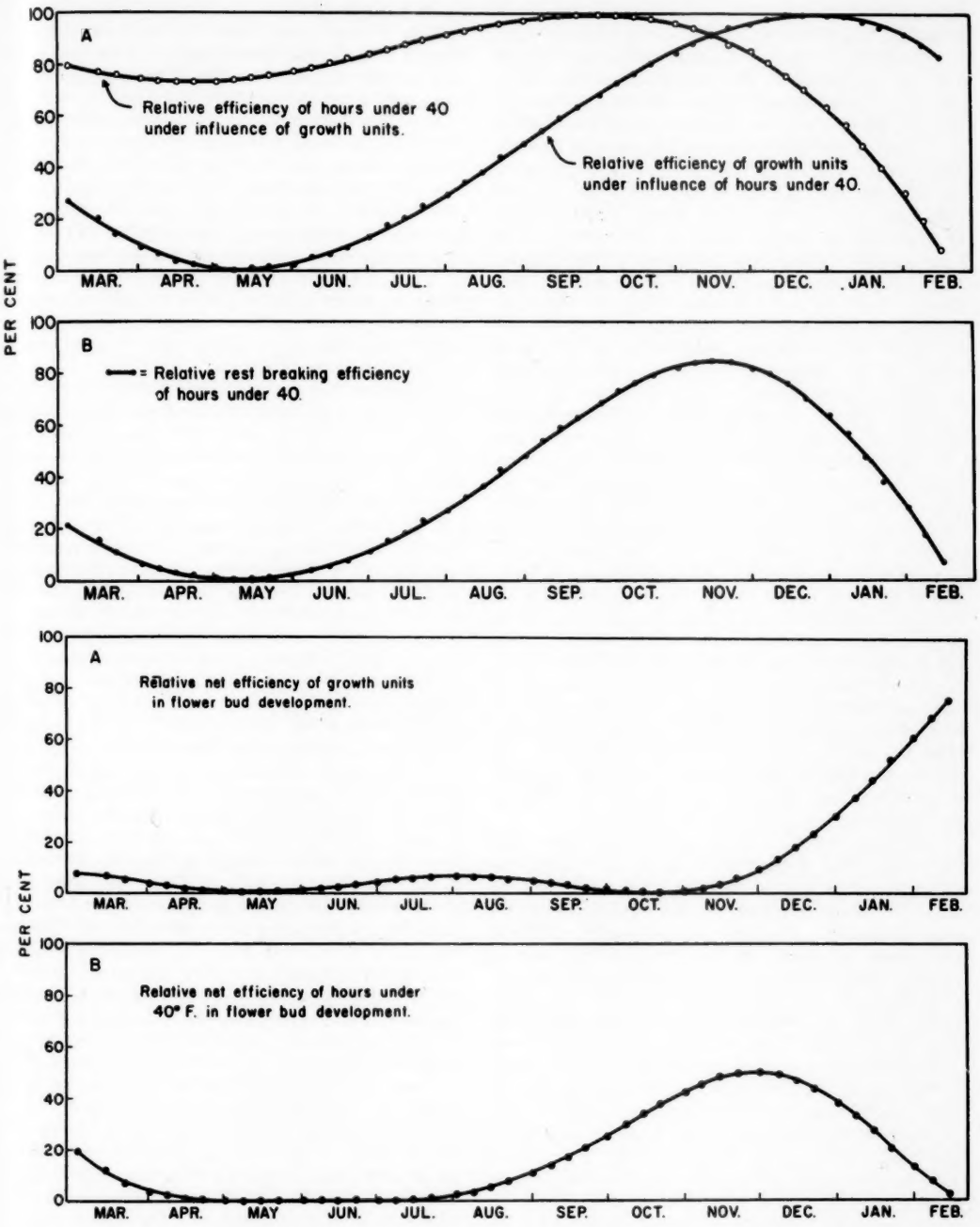

A
Relative efficiency of hours under 40 under influence of growth units.
Relative efficiency of growth units under influence of hours under 40.
PER CENT
100
80
60
40
20
0
MAR. APR. MAY JUN. JUL. AUG. SEP. OCT. NOV. DEC. JAN. FEB.
B
= Relative rest breaking efficiency of hours under 40.
MAR. APR. MAY JUN. JUL. AUG. SEP. OCT. NOV. DEC. JAN. FEB.
A
Relative net efficiency of growth units in flower bud development.
PER CENT
MAR. APR. MAY JUN. JUL. AUG. SEP. OCT. NOV. DEC. JAN. FEB.
B
Relative net efficiency of hours under 40° F. in flower bud development.
MAR. APR. MAY JUN. JUL. AUG. SEP. OCT. NOV. DEC. JAN. FEB.

FIG. 6 (*top*). A. Relative efficiency of growing temperatures (growth units) as altered by effects of chilling (plotted on dots) and relative efficiency of chilling temperatures (hrs under 40° F) as altered by growing temperatures (plotted on circles). B. Relative rest-breaking efficiency of chilling temperatures.

FIG. 7 (bottom). A. Relative net efficiency of growing temperatures (growth units) in flower bud development. B. Relative net efficiency of chilling temperatures (hrs under 40° F) in flower bud development.

in the different periods in terms of the degree to which the full or 100 % effectiveness is attained.

The curves in figure 5 B were treated like those in 5 A. In this instance, the comparison of the curves evaluates the modifying influence of growing temperatures on the relative effectiveness of hours under 40° F in advancing the time of bloom. In general, growing temperatures reduce the effectiveness of hours under 40° F from early spring to mid-June; the greatest reduction, −2.5, occurs in late April. From the end of June until the first of December, favorable growing temperatures increase the efficiency of hours under 40° F in shortening the time to bloom; the maximum benefits occur in early October when the difference between the curves is 5.9. From the first of December until full bloom, the effectiveness of the hours under 40° F is progressively decreased by the effects of growing temperatures, with a difference of −22.8 occurring for the February 16 to full bloom period. The differences between the curves in figure 5 B can also be transposed, like those in figure 5 A, to a comparative percentage basis; in this instance, they express the degree to which the efficiency of hours under 40° F, from period to period, throughout the season is modified by the influence of growing temperatures. Favorable temperatures depress the efficiency of hours under 40° F the most at full bloom; comparatively then, this represents the zero value with respect to the relative degree of effectiveness of the chilling hours. On the other hand, the efficiency of hours under 40° F is improved by growing temperatures the most in the week of October 2 to 8 when there is a positive difference of 5.9 between the curves; this difference represents the 100 % value. Other differences between the curves in figure 5 B were adjusted to values between 0 and 100 % and are shown graphically by the curve plotted on the circles in figure 6 A.

Two measures of chilling effectiveness have been presented. One, derived immediately above, rates the efficiency of chilling temperatures in shortening the time to bloom as altered by growing temperatures (fig 6 A, circles). The other, derived earlier, expresses the degree to which the rest-breaking influence of chilling temperatures modifies the growth unit effects; it is identical with the relative efficiency of growth units under the influence of hours under 40° F (fig 6 A, dots). The products of these two estimates of chilling effectiveness were calculated for each period in the season. The resulting values express the net efficiency of hours under 40° F in breaking the rest. For example, for the period Full Bloom to March 15, the relative efficiency of hours under 40° F as altered by growing temperatures is 79.8 % (fig 6 A, circles). However, the apparent rest-breaking efficiency as derived from the influence of hours under 40° F on growth unit effects is 26.6 % (fig 6 A, dots). Thus, if an hour below 40° F is only 79.8 % effective, the net relative rest-breaking efficiency must be the product of 0.798 and 26.6 or 21.2 %. The net relative rest-breaking efficiencies of hours under 40° F for all 46 periods throughout the season are plotted in figure 6 B.

Two expressions of the relative effectiveness of each have now been derived for the two variables, growing and chilling temperatures. Each expression characterizes a distinct feature of the seasonal distribution of a given variable's influence on time of bloom. The first expression for each variable is represented by the respective pairs of curves in figure 5, (A) for growth units and (B) for hours under 40° F. The influence of one variable is compared, one period with another, in terms of the relative effectiveness of the variable in shortening the time to full bloom. This evaluation weights the influence of the variable at a given time relative to its effectiveness in the period in which maximum (100 %) effectiveness for the season was attained. It expresses, therefore, the relative *intensity* of the effectiveness of a variable in the different seasonal periods.

There are two forms of this expression for each variable, the first independent of and the second as influenced by the effects of the other variable. In both forms, however, the different periods are compared by assigning relative values to the effects of growth units (fig 5 A) or of hours under 40° F (fig 5 B) on time of bloom as measured in the regression analysis; the curves are merely the transformation of each regression curve of figure 4 to a relative basis.

The differences between the members of each pair of transformed curves provide the basis for the second of the relative-effectiveness expressions derived for each variable. These are the relative-efficiency expressions represented by the curves of figure 6; they compare one seasonal period with another as to the degree to which the effects of one variable on time of bloom are modified by the influence of the other variable. The degree of influence of one variable on the other is not characterized by the individual curves of figure 5; only by evaluating the differences between them can the degree of influence be estimated. Such an evaluation weights the change in the effectiveness of one variable that can be attributed to the influence of the other; the change in effectiveness of the first variable in each period is related to the full (100 %) efficiency represented by the maximum positive (favorable) change induced by the second variable. The evaluation, therefore, characterizes the limitations imposed by one variable on the influence of the other during the different seasonal periods; it compares the degree of efficiency of the latter variable in the different periods. In this sense, it is an expression of the *quality* of a variable's effectiveness.

Taken together, the two expressions of a variable's effectiveness, its intensity and quality, afford a seasonal description of the net efficiency of its influence in advancing the time of bloom. Even more properly, they constitute a description of the net efficiency of the variable's influence in flower bud development, since time of bloom is a function of the rate of bud development. For each variable, then, a single expression of its net efficiency was derived for each seasonal period by combining the evaluations of its intensity and quality characteristics.

The intensity evaluations used were those which describe the effectiveness of one variable independently of the other (fig 5, circles). These constitute the basic seasonal patterns of effectiveness for the variables. The quality evaluations used were those shown by the curves for the efficiency of growth units (fig 6 A) and hours under 40° F (fig 6 B). These express the degree to which the basic pattern is modified in each of the periods. The net efficiency evaluations were obtained for each variable by multiplying the corresponding intensity and quality estimates of the variable's effectiveness. The results are plotted in figure 7 as (A) the relative net efficiency of growth units and (B) the relative net efficiency of hours under 40° F in flower bud development.

## Discussion

The development of the efficiency curves for chilling and for growth units plotted in figures 6 and 7 was an outgrowth of attempts to use the regression curves of figure 4 in interpreting the rest period. The regression coefficients plotted in figure 4 proved directly applicable only in predicting the time of bloom at successive intervals through the season. Such predictions, either by themselves or in relation to the long-time averages, did not yield much information on the rest period or the progress of buds through the rest in any given season. However, after the regression coefficients were transposed to the comparative percentages of figure 5, the seasonal effectiveness of growth units and of chilling could be evaluated. It was possible, then, to estimate the extent to which hours under 40° F reduce the effectiveness of growing temperatures in advancing time of bloom; the relative efficiency curve for growth units in figure 6 A is the result. Similarly, an estimate was possible of the extent to which favorable growing temperatures reduce the effectiveness of chilling temperatures; the relative efficiency curve for hours under 40° F in figure 6 A is the result. In addition, an evaluation of the effectiveness of hours under 40° F as the rest-breaking influence itself is also available in the efficiency curve for growth units (fig 6 A), considering that the maximum rest-breaking influence occurs when chilling hours interfere the least with growth unit efficiency and the minimum when they interfere the most. By combining the two evaluations of the effectiveness of chilling hours, the relative rest-breaking efficiencies of hours under 40° F as plotted in figure 6 B results.

In developing these expressions of the effects of temperature on time of bloom two distinct characteristics of each variable's influence are evaluated. The intensity of temperature influence in the different seasonal periods is expressed in the relative effectiveness curves of figure 5; the quality characteristic is expressed in the relative efficiency curves of figure 6. By combining these two evaluations, estimates are possible for the relative net efficiency of growth units and chilling hours in flower bud development (fig 7). These latter two curves are remarkable because they offer a quantitative expression of the response of buds to growing temperatures and to chilling which fits well with the behavior of buds as observed in the field. With the growth unit efficiency curve in figure 7 A, for example, a declining responsiveness to favorable temperatures is indicated from early March to early May. This is the period of initial shoot growth and bud formation. Apricot spur growth in particular is completed by mid-April so that a majority of the flower buds have been formed. The downward trend of the curve from March to May is in line with the observation that the buds gradually enter the rest and that they are fully under the rest influence by the time length growth (of spurs, in particular, for the apricot) has ceased. From late May until mid-October, favorable growing temperatures have some effect in stimulating bud development or advancement toward bloom, the maximum effectiveness for the period being in early August. The intensity of growth unit efficiency for this period is not great, but it parallels known bud behavior. On the average, the first microscopic signs of flower bud differentiation are detectable in apricots by mid-August. Flower parts continue to develop in the weeks following, with well-formed pistils and anthers being observable by mid-October. This type of bud development must be that which is promoted, at a low level of efficiency, by favorable growing temperatures during late summer. From early October to late November on the average, and in some seasons into December, there is little or no observable change in apricot flower buds. The anthers reach an early stage of sporogenous cell development sometime in October and remain at that stage until the rest influence is diminished. This phase of the average bud development picture coincides nicely with the low efficiency of growth units shown in figure 7 A for October and most of November. As the rest is broken in December and January the flower buds of apricot become increasingly more responsive to favorable temperatures, a behavior which is coincident with the increasing net efficiency of growth units indicated from December to full bloom in figure 7 A.

The curve for the relative net efficiency of hours under 40° F in figure 7 B has certain unique features, some of which are suggestive of factors not usually associated with the rest-breaking action of chilling temperatures. In the first place, chilling temperatures experienced during the period of shoot and spur growth in the spring appear to have some influence on the rest status of the buds. The net efficiency of hours under 40° F is about 20 % during the initial period in the spring; it declines thereafter but does not reach a minimum until early May, coincident with the time the buds are fully into the rest. Chilling during these early periods may well condition the buds so as to influence the depth of rest reached in the fall and the amount of total chilling required to end it.

From May to early August, the relative net efficiency of chilling is at a minimum. This results from the fact that both the intensity and quality of the effectiveness of hours under 40° F are at low levels

from May to August (figs 5 B and 6 B). Practically, of course, the effective summer values are low because normally no temperatures under 40° F are experienced during this period.

A second unique feature of the evaluation of chilling effectiveness (fig 7 B) is the peak which occurs in late November and early December. The timing of the peak is not unexpected; the importance of early December chilling, in particular, is well recognized. The magnitude (ca 50 %) is, however, surprisingly low to be the maximum efficiency value of the entire season. The low value is actually further evidence of the comparative importance of chilling in the spring during the early stages of bud development. The intensity of chilling effectiveness (fig 5 B) is greater in the early spring after bloom than during the following fall and winter. This relationship between spring and winter is reversed in the net efficiency evaluation of chilling (fig 7 B), only because the rest-breaking efficiency during the spring (fig 6 B) is relatively of a much lower quality than during the fall and winter.

The rapid rate of decline in the net efficiency of hours under 40° F from the early December peak is also rather surprising. It is a reflection of the fact that growing temperatures cause a rapid decline in the relative efficiency of hours under 40° F after late September (fig 6 A, circles). Chilling in December–January is potentially the most effective for breaking the rest; at least, during that period the relative efficiency of growth units as influenced by hours under 40° F is at a maximum (fig 6 A, dots). Under the modifying influence of growing temperatures, however, the rest-breaking efficiencies in the different periods become distributed as in figure 6 B; this distribution dominates the net efficiency evaluations in figure 7 B, and accounts for the declining net effectiveness of chilling from December to full bloom.

This late winter decline in the efficiency of chilling would not, at first glance, seem to fit in with Weinberger's observations of peaches (4). He noted that "intensive early-winter chilling hastened the rest-breaking processes and shortened the rest period, but a greater number of chilling hours was necessary to accomplish this." A similar situation with apricots has been experienced in California. The anomaly is in the need for a greater total number of chilling hours to end the rest period in mid-January than in mid-February. Chilling experienced in the two months prior to mid-January occurs at the highest net relative efficiencies, whereas that occurring later is less and less efficient. It would seem, then, that a greater number of hours would be necessary with the lower efficiencies late in the season than at the higher efficiencies of early winter. There are two arguments which oppose such a conclusion and which reconcile Weinberger's observations with the late winter decline in chilling efficiency.

In the first place, as the relative net efficiency of chilling declines, the net efficiency of growing temperatures increases (fig 7). Therefore, the growing temperatures which accompany moderate amounts of early chilling could result in a faster bud development than would obtain if the early chilling were more intense and not accompanied by growing temperatures. Moderate early chilling will gradually break the rest. Buds will be increasingly responsive to concurrently experienced growing temperatures and will advance in development toward bloom. Such bud development is evidence of continued emergence from the rest, so that at any given time the requirement for additional chilling could be less than if the chilling had been more intense or continuous and without the accompanying growing temperatures. In this way, the total number of chilling hours required to break the rest might be reduced; the time required to effect the break, however, could be longer than with more intense, high efficiency chilling.

A second possible explanation of the anomaly concerning the declining chilling efficiency and the total chilling requirement is found in the method of deriving the relative efficiency values. The initial regression analysis constitutes an evaluation of the seasonal temperature distribution in relation to time of bloom. The method involves the calculation of coefficients which measure different characteristics of the temperature distribution. The correlation of the distribution coefficients with time of bloom is a part of the analysis. The simplest of these coefficients measures the relation of the total seasonal temperature experience to time of bloom. The others represent evaluations of successively higher polynomials fitted to the distribution pattern. The correlation of the seasonal totals of either temperature variable with time of bloom was very low and insignificant. The best correlations were with the distribution coefficients of the second and third degree; it was for this reason that a third degree polynomial was used to represent the regression. The fact that the distribution of the temperatures, not the seasonal totals, gave the best evaluation is pertinent to the explanation of the point in question. It suggests that either temperature variable is most effective when its experience is optimally proportioned among the different periods of the season. This could mean that in a season which started out with early and intense chilling, the chilling in each successive period would need to be proportionately as high as the preceding amounts, if the expected efficiencies were to be attained. On the other hand, in a season which started out with moderate chilling, the amounts in the successive periods could be proportionately at a comparably lower level. In this latter situation, a longer time might be required to break the rest, but the total chilling accumulation could actually be smaller than in a season of early and more intense cold.

Observations in the field and with trees subjected to controlled chilling give some support to both explanations of the anomaly raised by Weinberger's observation. For example, although Bennett (1) has reported on the slowness of Hardy pear trees to emerge from the rest when chilling is periodically interrupted by exposure to growing temperatures, in subsequent,

unpublished work (1953), he has demonstrated that exposure to the proper proportions of alternate chilling and growing temperatures may result in faster emergence from the rest than with continuous chilling. Neither of the arguments presented above affords by itself sufficient explanation of the situation. If taken together, however, they complement one another and constitute a reasonable explanation, one which gives further insight into the relationship of temperature experience to bud development.

One of the objectives in evaluating the rest period has been the developmnt of a chilling index that would be more critical and reliable than simple summations of hours under 45° F or under 40° F. This objective has not yet been attained. Efforts to characterize the known seasons with and without typical mild winter troubles have not resulted in a simple, precise means of distinguishing between them. Actually, the net efficiency expressions shown in figure 7 are an outgrowth of the fact that the regression coefficients of figure 4 were unsatisfactory for characterizing the known seasons. The net efficiencies were the result of efforts to derive factors from the regressions which would be more precise in describing the rest period. When the efficiency factors are used to weight the effectiveness of chilling and of growth units, simple summations of the weighted values offer no particular advantages over other indices. Reasonable intepretations and comparisons of the different years are possible by manipulation of the weighted values, but still no one simple criterion has been derived. One further, promising possibility is being explored. Satisfactory predictions and evaluations in unknown seasons may be possible from a regression of hours under 40° F (or under 45° F) on the seasonal distribution of the values for growth units and for hours under 40° F weighted by the efficiency factors of figure 7.

## Summary

The rest period of Royal apricot flower buds was evaluated by relating the time of bloom to the temperatures experienced throughout the preceding season. Two temperature expressions were used. One was in terms of growth units, an expression of temperature favorability which weights the effectiveness of different temperatures. The other was the expression of chilling experienced as hours under 40° F.

One evaluation was made by plotting the simple correlation coefficients derived from the relationship between time of bloom and the growth units per day experienced from selected dates to full bloom; the dates were scattered throughout the entire season from one full bloom to the next. The resulting curve indicated that on the average the rest influence in the buds gradually deepens after late June, reaches a maximum in late October and early November, and declines rapidly thereafter, so that by mid-January buds are quite responsive to growing temperatures.

This conclusion was confirmed by another, more critical evaluation which permitted an analysis of the influence of both growing and chilling temperatures on bud development. It was based on third degree regressions of the time of bloom on each of the two temperature variables. These regressions described the effect on time of bloom of a unit change in the temperature variable for 46 different periods during the season from full bloom to full bloom.

The regression curve for growth units indicated that the rest is deepest in October and that thereafter the buds emerge rapidly from the rest. The curve for hours under 40° F indicated that chilling in November, December, and January was particularly effective in breaking the rest and, thus, in shortening the time to bloom; in addition, chilling during the few weeks in early spring when new buds are forming had a marked effect in advancing the time of bloom in the following spring.

A regression analysis which combined the two variables was also made. This permitted an evaluation of the effects of each variable independent of the influence of the other. Estimates were derived from the several regression curves, expressing the relative intensity and efficiency (quality) of each variable's effectiveness in the different seasonal periods in furthering bud development and advancing time of bloom. These expressions are in accord with the known patterns of bud development and with the known relationships between temperatures and the rest period. They constitute a quantitative description of the relation of temperatures to those phenomena.

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# STUDIES ON TOBACCO ALKALOIDS. II. THE FORMATION OF NICOTINE AND NORNICOTINE IN TOBACCO SUPPLIED WITH $N^{15}$ [1, 2]

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The preceding paper (11) reports the changes in nicotine and nornicotine content as related to growth conditions of two selections of Nicotiana plants which contain principally nornicotine. Certain results obtained proved difficult to explain on the basis of current theories which state that nicotine synthesis is conducted predominantly in the root (4) and subsequent conversion to nornicotine takes place only in the leaf (5). It appeared that some of the uncertainties might be cleared up by means of experiments employing $N^{15}$.

## MATERIALS AND METHODS

A common variety of Maryland tobacco, *Nicotiana Tabacum* L. var. Robinson Medium Broadleaf, and of tomato, *Lycopersicum esculentum* Mill. var. Rutgers, were used in these experiments. Plants of these varieties were used as intact individuals and also after tongued inarch grafting of each scion on the other stock. In some instances grafts of tobacco on tobacco and tomato on tomato were also made in an effort to distinguish between the anatomical or mechanical effects of grafting and the physiological effects due to presence of unlike tissue.

The plants were grown in solution culture using McMurtrey's $C_1$ solution (7) modified as necessary to use $N^{15}$. Of the 225 ppm of N in this solution, 150 ppm were derived from $KNO_3$ and $HNO_3$, each of which contained 30 atom percent excess $N^{15}$. Thus, the nutrient solution contained about 45 ppm of $N^{15}$ as nitrate, or about 20 atom percent excess $N^{15}$. All the nitrogen in this solution was applied as nitrate. Detached leaf culture following the techniques of Dawson (3) was also employed.

Tobacco samples were treated as indicated in table I. Nicotine was obtained from the fractional steam distillate using MgO. The nicotine was purified by picrate precipitation but nornicotine was not purified satisfactorily from the distillation residue by picrate precipitation, due to the considerable amounts of other alkaloids present. A partial purification was conducted by distillation with steam from 5 % NaOH and extraction of the distillate with ethyl ether. Since this solution was still impure, a new method of purification of nornicotine was developed in which the Craig countercurrent apparatus (2) was used. In the course of this development the separation of a mixture prepared from nicotine, nornicotine, and anabasine was investigated by means of this apparatus,

TABLE I

SEPARATION OF FRACTIONS FROM TOBACCO SAMPLES

A. Green sample, 10 gm green tissue
  1. Extract with 50 % (final conc) acetone
  2. Filter
  3. From one portion of filtrate, determine total alkaloid by distilling with NaOH and NaCl and make spectrophotometric analysis on distillate (10)
  4. From another portion, determine nicotine and nornicotine by paper chromatography, elute spots and make spectrophotometric analysis of each (10)

B. Remainder of green sample—extract with 0.1 *N* HCl
  Residue—keep in 80 % alcohol for amino acid study
  Extract—concentrate, neutralize, fractionally distill from MgO
    Distillate
      1. Concentrate
      2. Make nicotine picrate
      3. Recrystallize
      4. Dissolve in 5 % NaOH
      5. Steam distill
      6. Check spectrum for purity
      7. Kjeldahl
      8. Oxidize $NH_3$ to $N_2$
      9. Analyze with mass spectrograph for $N^{15}$
    Residue
      1. Make to 5 % NaOH
      2. Steam distill
      3. Make distillate to 5 % NaOH
      4. Extract into ethyl ether
      5. Separate nornicotine by countercurrent distribution
      6. Check fractions by paper chromatography
      7. Combine nornicotine fractions
      8. Make to 5 % NaOH
      9. Steam distill
      10. Check purity spectrophotometrically
      11. Kjeldahl
      12. Oxidize $NH_3$ to $N_2$
      13. Analyze with mass spectrograph for $N^{15}$ derived from nornicotine

using various organic solvents and buffers of various pH values saturated with each other. The results of certain of these experiments are shown in figure 1. The best separation of nornicotine from nicotine and anabasine, and also from other constituents of tobacco extracts, was found with chloroform as a stationary phase and 0.2 M $KH_2PO_4$-NaOH buffer of pH 7.0 as an aqueous moving phase. After 30 transfers under these conditions nearly all of the nornicotine had passed tube 20, whereas none of the impurities determinable by paper chromatography (10) had done so. The picrate of nornicotine which had been separated in this manner was found to have no depression of mixed melting point with authentic nornicotine picrate.

Nicotine and nornicotine which had been purified

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[2] Contribution no. 2713, Maryland Agricultural Experiment Station, Scientific Article A 559.

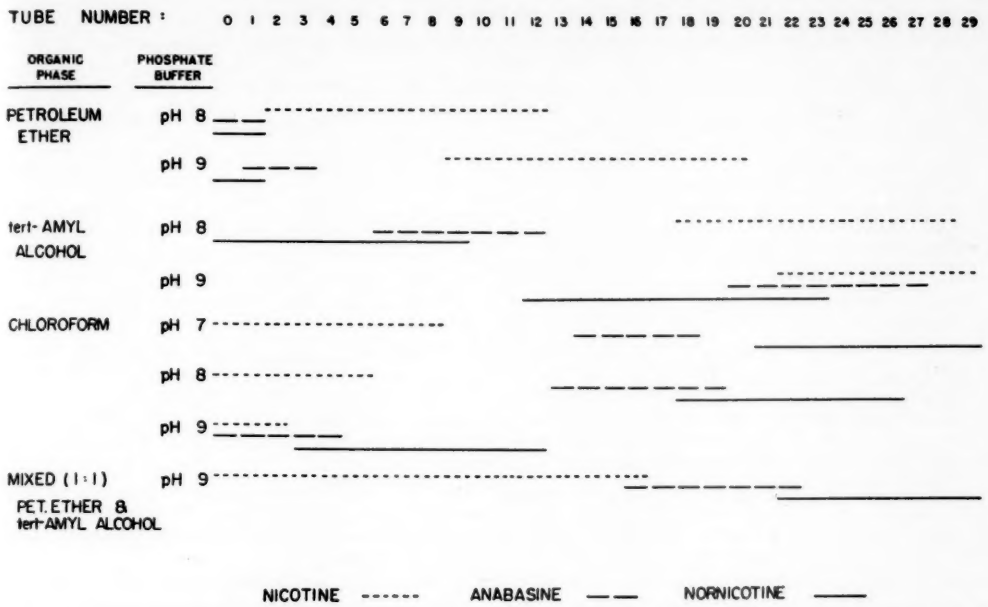


FIG. 1. Countercurrent distribution of nicotine, nornicotine, and anabasine. The organic phase moves and the aqueous buffer is stationary except when chloroform is used. The best separation was obtained with chloroform and pH 7 buffer.

from tobacco extracts, as above, were each converted to ammonia by the Kjeldahl method and the ammonia was oxidized to nitrogen with hypobromite and measured with the mass spectrometer as described by Rittenberg (8).

## EXPERIMENTS AND DISCUSSION

PRELIMINARY $N^{15}$ EXPERIMENT: The use of $N^{15}$ can provide proof of the course of chemical reactions only in cases where it can be proven that exchange of nitrogen within the compound in question does not occur (1). Since exchange of nitrogen in other compounds has been reported (13), a preliminary experiment was necessary to prove that this did not occur in the pyridine alkaloids.

Nicotine was supplied to tomato plants in regular solution culture by adding one gram of nicotine per liter of the nutrient solution and readjusting the pH. Analysis showed that nicotine went into the tomato plants, as such, and built up an alkaloid concentration similar to that in tobacco plants at the corresponding growth stage. These $N^{14}$ nicotine-containing tomato plants were transferred into a nicotine-free nutrient solution, which contained 20 % $N^{15}$ excess in the form of nitrate, where they grew for two more weeks. They were then harvested and analyzed as shown in table II. It will be noted that no pyridine alkaloids were found in the tomato plant before nicotine was supplied, which may be considered to be at variance with the findings of Wahl (12). Actually, Wahl claimed a nicotine content of 1 to 2 mg per 100 gm dry substance, or about 1 to 2 ppm of fresh tissue. Since the average fresh weight of the tomato plants at this stage was 33 gm, this would correspond to 0.03 to 0.07 mg per plant which would be determinable without the rigorous concentration procedures used by Wahl. In 4 years' work we have never found nicotine in the strain of tomato plants here used, but 1 ppm would be near the limit of detection by our procedure.

The difference in nicotine content before and after transfer of the tomato plants from the nicotine-containing nutrient solution (table II, (c) vs (d) and (e)) is large, probably due to low values for (c), but the small quantity of $N^{15}$ found in the nicotine of the plants grown in 20 % excess $N^{15}$-containing nutrient solution for two weeks makes it seem improbable that any significant synthesis occurred. In two independent samples only 0.006 % and 0.007 % excess of $N^{15}$ over the average natural abundance of 0.367 atom percent were found in nicotine isolated from the tomato plants of this experiment. An ammonium sulfate blank analyzed 0.004 % excess $N^{15}$ and nicotine isolated from a tobacco plant grown in $N^{14}$ nutrient culture analyzed 0.004 % excess. The excess $N^{15}$ found in the nicotine from tomato plants was not much greater than that found in the blank samples. Thus evidence of substitution of $N^{15}$ for $N^{14}$ in nicotine was not obtained. Since such substitution is presumably a property of the compound rather than of the plant, one would expect the same results in the tobacco plant if a similar experiment could be performed with it.

PRELIMINARY GRAFTING EXPERIMENT: In order to use grafting techniques in connection with the investigation of the synthesis of alkaloids in the plant, it is

TABLE II

EXPERIMENT ON THE POSSIBLE SUBSTITUTION OF NITROGEN IN TOBACCO ALKALOIDS
(MG ALKALOID/PLANT)

| SAMPLE (WHOLE TOMATO PLANT) | TOTAL ALKALOID * | NICOTINE | NORNICOTINE |
|---|---|---|---|
| a. Before nicotine feeding | Not found | Not found | Not found |
| b. Grown 3 days in nutrient solution containing $N^{14}$ nicotine (1 gm/l) | 19 | 19 | Not found |
| c. Grown 2 wks in nutrient solution containing $N^{14}$ nicotine (1 gm/l) | 40 | 40 | Not found |
| d. Plant from c transferred to nicotine-free, $N^{15}O_3^-$-containing nutrient solution and grown 2 additional wks | 79 | 66 | 9.5 |
| $N^{15}$ excess | .. | *0.007 %* ** | .. |
| | .. | *0.006 %* *** | .. |
| e. Plants from c transferred to $N^{14}$ nutrient solution and grown 2 additional wks | 71 | 62 | 7.4 |

* Including other alkaloids besides nicotine and nornicotine.
** Reference sample 1—Regular nicotine from tobacco ($N^{14}$ culture), 0.004 % excess $N^{15}$.
*** Reference sample 2—$(NH_4)_2SO_4$, 0.004 % excess $N^{15}$.

necessary to know the extent to which the presence of the graft union influences the physiological processes of the plant. Few experiments of this kind are described in the literature and none under the experimental conditions used here. Consequently, preliminary experiments were conducted in which plants of tobacco grafted on tobacco and of tomato grafted on tomato were compared with intact plants of the same strains. The tobacco plants were grown in nutrient solution (7) and the tomato in this plus 0.5 gm of nicotine per liter. The growth of the plants was interrupted by the injury resulting from grafting, so the grafted plants were not as large and did not contain as much nicotine as the intact plants of the same age. However, each part of these plants contained about the same amount of alkaloids as the corresponding part of younger intact plants of the same size. Thus it appears that grafting within a given strain does not have sufficient effect on the ability of these strains to synthesize, translocate, or degrade these alkaloids to be detected in the presence of the general inhibitory effect of the graft injury.

The above-described experiment concerns the mechanical effects of grafting but does not concern the phenomenon of graft incompatibility between different plant species. In an effort to measure this effect between the strains involved, plants were grown consisting of roots and tops of tobacco with tomato stems in between, and similar ones containing roots and tops of tomato and intervening tobacco stems. Both types were grown in nutrient solution without added nicotine. In these "sandwich" plants the alkaloid content of the various parts was quite variable from plant to plant. The difference in alkaloids between the twice-grafted plants, part for part, and ungrafted plants of the same size, and bearing the same kind of root, is not great in most cases. Thus the evidence of significant graft incompatibility influencing the translocation of nicotine or possible precursors across the union was not obtained.

GRAFTING EXPERIMENTS USING $N^{15}$: Plants composed of tobacco shoots grafted on tomato roots were used principally. Samples were taken 1) at the time of grafting, 2) six weeks later, just before transplanting the grafted plants into $N^{15}$ solution, and 3) after four weeks in $N^{15}$ solution, at normal harvesting maturity. Corresponding samples of intact tobacco and tomato plants grown under similar conditions were harvested at the same times for reference. Table III shows the results expressed as mgs of alkaloid found per plant part. Analyses were made on combined samples from four to six plants, so as to have enough material for $N^{15}$ determinations.

At the time of grafting 3.7 mg of total alkaloid were found in the tobacco roots and 13.4 mg in the tobacco shoots. Since the tomato plants contained nothing reported as any pyridine alkaloid by the methods used, the initial alkaloid content of the grafted plants composed of tobacco scions on tomato roots was also approximately 13.4 mg. Six weeks later, when some of the grafted plants were transferred from the nutrient solution containing $N^{14}$ to that containing approximately 20 atom percent excess $N^{15}$ as nitrate, the total alkaloid content of the tomato stock was negligible, and that of the tobacco scion of these same plants was 16.2 mg. Since the grafted plants were not cut loose from the tobacco roots until a few days after the grafts were made, the increase during this period is not considered to be significant. However, in the four weeks following the transfer to $N^{15}$ the total alkaloid content rose to about four times the level at grafting and the nornicotine level was proportionately more. The alkaloid increase in the plants transferred to new $N^{14}$ nutrient solution was not so great, but was still two to three times. Since great care was taken to see that the tobacco scion was not permitted to develop roots, and

none were found inside the graft union, the conclusion appears to be inescapable that nicotine alkaloids were synthesized in these plants composed of tobacco scion and tomato stock. Further proof of this is provided by the mass spectrographic analysis of the $N^{15}$ excess in nicotine and nornicotine purified from these plants at harvest. Each of two nicotine samples contained nearly 6.5 atom percent excess $N^{15}$ and a nornicotine sample contained about 6.2 %.

Admittedly, the $N^{15}$ content of the alkaloids indicates that not more than one-half of the excess was found which would be expected if $N^{15}$ had been incorporated in the proportion of 20 % excess in which it was present in the nutrient solution into all of the increased amount of nicotine found subsequent to transfer into $N^{15}$ enriched solution. Thus it appears that more than one-half of this increased nicotine found after transfer to the $N^{15}$ enriched solution may have been formed from nitrogen compounds without excess $N^{15}$, and so presumably was present in the plants at the time of transfer; but the remainder appears to have been formed from nitrogen which was present as 20 % excess $N^{15}O_3^-$ in the solution into which the plants composed of tomato root and tobacco top were transferred.

The increase, during ten weeks, of the alkaloid content in the two lots of grafted plants with tomato roots was only about 43 and 25 mg, whereas the increase in the two grafted lots with tobacco roots was 92 and 166 mg, and in the intact tobacco plants 344 mg. So it is clear that the majority of the alkaloid synthesis normally takes place in tobacco roots, but it also appears that synthesis through all the steps from nitrate in the nutrient solution to nicotine or nornicotine can take place in plants composed of tobacco scions on tomato stock. Since it seems likely that amino acids or closely related compounds form a part of the chain of precursors of nicotine, and they are probably formed by the tomato plant as well as the tobacco plant, we cannot say on the basis of present information which steps can take place in which part of the plant. Evidently some critical step ordinarily takes place principally in the tobacco root and much of the synthesis of alkaloid is probably completed there.

Since the $N^{15}$ excess in the nutrient solution of certain lots was 20 atom percent during the last four weeks of the experiment and no excess $N^{15}$ existed in the grafted plants before that period, one would expect that 1) a higher percentage of $N^{15}$ excess would exist in the alkaloid being formed most extensively during this period as compared to the earlier period, and 2) in the case of a given alkaloid a greater $N^{15}$ excess would exist in the plant part in which it was synthesized than in the plant part to which it was being translocated. The results here presented show a higher percentage excess of $N^{15}$ in the nicotine from the tobacco root of the grafted plant than in the tomato shoot, confirming the previous workers' findings as to the direction of translocation of the majority of the nicotine. However, they also show a higher percentage excess of $N^{15}$ in the nornicotine from the root of these plants (9.4 atom %) than in the nicotine from the root (6.4 %), or the nicotine (5.3 %), or nornicotine (7.5 atom %) from the shoot. This cannot be explained on the basis of demethylation of

TABLE III

ALKALOID CONTENT (MG/PLANT PART) AND PERCENTAGE EXCESS $N^{15}$ IN THE ALKALOIDS OF GRAFTED AND UNGRAFTED PLANTS

| SAMPLE | ROOT | | | SHOOT | | |
|---|---|---|---|---|---|---|
| | TOTAL ALKALOIDS * | NICOTINE | NORNICOTINE | TOTAL ALKALOIDS * | NICOTINE | NORNICOTINE |
| *Intact plants* | | | | | | |
| Tobacco | | | | | | |
| At grafting time | 3.7 | 3.0 | 0.4 | 13.4 | 10.5 | 0.9 |
| At harvest | 90.5 | 69.5 | 17.2 | 271.0 | 215.0 | 48.8 |
| Tomato | | | | | | |
| At grafting time | 0 | 0 | 0 | 0 | 0 | 0 |
| At harvest | 0 | 0 | 0 | 0 | 0 | 0 |
| *Grafted plants* | | | | | | |
| Tobacco scion on tomato stock | | | | | | |
| At time of transfer to new solution | 0.3 | 0.2 | 0 | 16.2 | 11.5 | 2.1 |
| After 4 wks in $N^{15}$ solution | 0.5 | 0 | 0 | 55.6 | 40.9 | 8.9 |
| $N^{15}$ excess in the alkaloids | ... | .... | .... | .... | *6.49 %* | *6.22 %* |
| | ... | .... | .... | .... | *6.50 %* | .... |
| After 4 wks in new $N^{14}$ solution | 0.4 | 0 | 0 | 38.2 | 28.7 | 7.3 |
| Tomato scion on tobacco stock | | | | | | |
| At time of transfer to new solution | 6.8 | 4.8 | 1.9 | 16.7 | 14.9 | 1.3 |
| After 4 wks in $N^{15}$ solution | 14.8 | 12.4 | 1.9 | 80.6 | 60.1 | 12.5 |
| $N^{15}$ excess in the alkaloids | ... | *6.43 %* | *9.38 %* | .... | *5.30 %* | *7.49 %* |
| After 4 wks in new $N^{14}$ solution | 28.8 | 22.2 | 5.4 | 141.3 | 106.0 | 25.8 |

* Including alkaloids besides nicotine and nornicotine.

nicotine to nornicotine in the shoot and translocation back to the root. Also, it cannot be explained as resulting demethylation of root nicotine. It would appear from the results that the root nicotine might have been derived from root nornicotine and that in this experiment most of the leaf nornicotine arose by translocation of root nornicotine rather than by demethylation of shoot nicotine. These data do not prove that nicotine was formed from nornicotine in the root, but they show that the majority of the nornicotine could not have come from nicotine under the conditions of the experiment. The quantity of root nornicotine did not increase appreciably during the last four weeks of the experiment, which is not surprising if it was being used up both by methylation to nicotine in the root and by translocation as nornicotine to the shoot.

Alkaloid Changes During Air-Drying: In order to study this problem further, two intact Robinson plants were grown in the same manner as the checks of the previous experiment, except that they were grown in about 20 % excess $N^{15}$ solution in the form of nitrate for the last four weeks. Sampling was done as in part 3a of the preceding paper (11). That is, samples for immediate analysis consisted of 1) alternate leaves from each of two plants, 2) one longitudinal half of the stem of each, and 3) one longitudinal half of the roots of each. The other sample of each plant part was composed of the remaining halves of both plants after drying for one week in the greenhouse. The results are shown in table IV.

The amount of both nicotine and nornicotine found in the root and stem samples, which had been dried for a week, was much less than in the initial amount but the proportion of each to the total alkaloid content was not changed significantly. However, in the leaf the total alkaloid content did not decrease significantly, but the majority of the nicotine was converted to nornicotine. Thus the present experiment confirms previous workers in showing that the conversion of nicotine to nornicotine occurs in the leaves but does not occur to an appreciable extent under these conditions in the stalk or root.

In addition to the above-mentioned data, the amount of excess $N^{15}$ in the purified alkaloids of the leaf is shown in table IV. Two samples of the purified initial leaf nicotine were separately Kjeldahlized and analyzed by mass spectrography to give some indication of the reproducibility of results. There was not sufficient $N^{15}$ alkaloid available to permit replication of all samples. The percentage excess $N^{15}$ does not appear to be significantly different between the initial nicotine and nornicotine samples and the nicotine sample after a week's drying. However, the nornicotine sample at the later date appears to be appreciably higher in $N^{15}$. The only explanation of this increase, if real, which has been proposed so far is that additional nornicotine was synthesized from precursors which were not steam distillable or had no ultraviolet absorption maximum near 259 $m\mu$, but which were present in the leaves when detached. Though this explanation cannot be verified at this time, it may be noted that a decrease of 10 to 20 % in alkaloid on a per plant basis usually occurs during curing of tobacco and decreases of 39.5 and 28 % were observed in the root and stem in this material, whereas a decrease of only 1.2 % was observed in the case of this leaf sample. The increase in $N^{15}$ excess from about 6 % in the other samples to about 9 % in the leaf nornicotine during air-drying could be explained if we could assume the synthesis from precursors containing 20 % excess $N^{15}$ of an amount of nornicotine equal to 18 % of the amount of alkaloid present. This would also explain the failure to observe significant loss of total alkaloid in the leaf. It would also be in line with results, presented below, on detached leaf culture.

Detached Leaf Culture: Table V shows the results of an experiment designed to investigate further the ability of detached leaves to synthesize alkaloids. The leaves of two tobacco plants which had been grown in regular $N^{14}$ culture solution were used. Alternate leaf samples were taken in the same way as in the previous experiments, One group, 276 gm, was used for green-sample analysis, the other group, 272 gm, for detached leaf culture. The petiole of each leaf in the latter group was inserted into a nutrient solution containing 20 % excess $N^{15}$ in the form of nitrate. The growth of micro-organisms in the nutrient solution was minimized by immersing the container in cold running water as suggested by Dawson

TABLE IV

THE TRANSFORMATION OF NICOTINE TO NORNICOTINE DURING AIR-DRYING
(MG ALKALOID/PLANT PART)

| Plant part | Initial content | | | Content after 1 wk air-drying | | |
|---|---|---|---|---|---|---|
| | Total alkaloid | Nicotine | Nornicotine | Total alkaloid | Nicotine | Nornicotine |
| Root | 49.2 | 38.3 | 8.8 | 29.8 | 24.5 | 4.1 |
| Stem | 37.9 | 32.5 | 4.0 | 27.4 | 22.6 | 3.6 |
| Leaf | 110.3 | 83.8 | 20.0 | 109.0 | 17.4 | 79.3 |
| | | *Excess $N^{15}$ in leaf alkaloids* | | | | |
| % | .... | *7.05* | *6.25* | .... | *6.26* | *9.24* |
| Mg | .... | 1.02 | 0.24 | .... | 0.19 | 1.39 |
| % | .... | *6.80* | .... | .... | .... | .... |
| Mg | .... | 0.99 | .... | .... | .... | .... |

TABLE V

CHANGES IN ALKALOID CONTENT RESULTING FROM ONE WEEK OF DETACHED LEAF CULTURE

| | TOTAL ALKALOID CONTENT * | NICOTINE | NORNICOTINE |
|---|---|---|---|
| | *mg* | *mg* | *mg* |
| Original alkaloid content | 614 | 489 | 90 |
| After 1 wk, detached leaf culture in $N^{15}$ solution | 688 | 534 | 128 |
| Excess $N^{15}$ | ... | *0.010 %* ** | *0.010 %* ** |
| | ... | *0.009 %* *** | .... |

* Including alkaloids other than nicotine and nornicotine.

** Reference sample 1—Regular nicotine from tobacco in $N^{14}$ culture, 0.004 % excess $N^{15}$.

*** Reference sample 2—$(NH_4)_2SO_4$, 0.004 % excess $N^{15}$.

(3). Compressed air was also supplied into the solution, as was done with rooted plants.

Leaf samples were analyzed after one week of detached leaf culture. The results are expressed as mg alkaloid per sample corresponding in size to the average number of leaves per plant. The total alkaloid content in the leaves increased from 614 mg to 688 mg, or 12 %. The nicotine content increased from 489 mg to 534 mg, a 9 % increase. The nornicotine content increased from 90 mg to 128 mg, an increase of 42 %. However, only 0.010 and 0.009 % of $N^{15}$ excess was found in nornicotine. These observed excess $N^{15}$ figures are higher than the percent $N^{15}$ excess found in the N-atom substitution experiment, but cannot be considered to be significant since the reference natural-abundance samples averaged an "excess" of 0.004 % $N^{15}$. Thus they do not constitute proof of the formation of alkaloids by complete synthesis from $N^{15}$ nitrate in detached leaf culture. Contamination of the "purified" alkaloids with 30 ppm of a nitrogen-containing compound of about the same molecular weight but containing 20 % excess $N^{15}$ would result in the average observed excess found in these three alkaloid samples over that found in the two reference samples. On the other hand, the increase in amount of total and individual alkaloids can hardly be explained as due to sampling error. The leaves in each sample, with almost identical initial weight, came from the same plants and a marked increase in nicotine, nornicotine, and independently determined total alkaloid was observed following detached leaf culture. These results agree with the indications obtained in the air-drying experiment, that tobacco alkaloids are formed through intermediate nitrogen-containing compounds or precursors.

In view of what is now known of intermediary metabolism, we would suspect that the synthesis of any molecule as complex as nicotine or nornicotine would occur in a number of steps. These reactions may occur principally in different parts of the plant. The experimental techniques formerly available did not permit one to distinguish between these steps. Dawson has utilized the evidence that small quantities of nicotine are present in the root tip together with the evidence from his grafting experiments to assert that nicotine is synthesized in the roots. The results presented here could be explained on the assumption that some $N^{14}$ nitrogen had been taken up by the roots of the plant and had undergone the critical transformation into potential alkaloid-forming, nitrogen-containing compounds which were present at least in the leaves; but, in this case, the final conversion to alkaloids was not completed until after the leaves were removed from the stem, during the period of detached leaf culture.

## CONCLUSION

Results presented here, which were obtained by employing $N^{15}$, tend to confirm and extend those reported in Part I of this paper (11). These results indicate that the synthesis of nicotine alkaloids follows a stepwise course involving a number of reactions and possibly alternate pathways. They appear to proceed through nitrogen-containing precursors which do not react as alkaloids to the analytical methods used in these experiments. In the case of plants growing under normal conditions it is evident that the rate-limiting step of this synthesis is carried out in the root more extensively than elsewhere. It is also evident that the synthesis of some of the nicotine is completed there. This is in line with the previous findings of Dawson (4) and many others. However, evidence is also presented that nicotine may be synthesized in the detached leaf, but if this occurs it is not a complete synthesis from inorganic nitrogen but is a conversion of nitrogen-containing compounds from a form in which they do not react to the analytical methods used. These methods are sensitive to most pyridyl compounds.

Nornicotine is generally known to be a minor constituent of the alkaloid fraction of many strains of *N. Tabacum* and a major constituent of certain strains of this species (6) and of most of the other species of Nicotiana which have been studied (9). It has been stated (5) that it is produced only in the leaf at the expense of nicotine. The present experiments confirm previous observations that nicotine can be converted to nornicotine in the leaf. However, since nornicotine was detected in the root at an early stage of seedling growth, as described in the previous paper (11), and was found in the work reported here to contain a higher percentage excess $N^{15}$ than the nicotine in plants shifted into $N^{15}$-containing nutrient culture during their later growth, it appears that nornicotine can be formed independently either in root or in leaf of tobacco plants.

If the synthesis of nicotine passes through nornicotine some of the results can be explained. In that case the nornicotine of most strains of tobacco appears to remain, during the early stages of growth, at a level below that which is detectable by the methods which have been used in the past. In the later stages of growth both of the strains used in this work devel-

oped a high nornicotine content. Much of this nornicotine derives from the nicotine present in the leaves, but as shown in the previous paper the nornicotine content may continue to rise after nicotine is no longer detectable. This evidence, combined with the higher $N^{15}$ content of the isolated nornicotine, requires the consideration of the possibility that some of the nitrogen may be synthesized into organic precursors of the alkaloids in the roots and then into nornicotine without having been converted to nicotine. The present results do not indicate whether translocation from the roots to the leaves of these predominantly nornicotine-containing strains occurred principally as nornicotine or as a precursor of it.

The differences in results obtained in these experiments from those of other workers may be explained as follows:

a) The use of an ultraviolet spectrophotometric method for total alkaloid determination and of paper chromatographic and countercurrent distribution methods for alkaloid separation and identification permits the estimation of much smaller quantities, especially of nornicotine in the presence of nicotine.

b) The use of $N^{15}$ permits one to distinguish nitrogen taken in after a change in culture solution from that taken in before.

c) Different strains of plants were selected to make the changes involving nornicotine more pronounced so that they could be more readily detected.

d) Different techniques were used in the growth of the plants.

The results of these experiments provide evidence of a much more complicated set of reactions involved in the synthesis and transformations of the tobacco alkaloids than have been recognized previously, but much more data will be required before the course of all the reactions can be defined. Further studies on the alkaloid precursors and on the possibility of methylation of nornicotine to nicotine in tobacco plants will be helpful in understanding these reactions.

## Summary

1. A study of the formation of nicotine and nornicotine has been conducted employing tongued inarch grafting, detached leaf culture, and isotopic nitrogen ($N^{15}$) as nitrate in nutrient solution culture. *Lycopersicum esculentum* Mill. var. Rutgers and *Nicotiana Tabacum* L. var. Robinson Medium Broadleaf, a tobacco variety known to contain relatively large proportions of nornicotine, were used.

2. Nicotine synthesis in plants composed of tobacco scions on tomato stock was observed in far larger quantities than would be expected on the basis of previous studies. Grafted plants were transferred to a nutrient solution containing 20 % excess $N^{15}$ as nitrate. After four weeks' growth in this solution the alkaloids isolated from these plants contained about 6 % excess $N^{15}$.

3. In plants composed of tomato scions on tobacco stocks, grown in $N^{15}$ nutrient solution after grafting, the nornicotine isolated from the roots contained a higher $N^{15}$ excess than did the nicotine, indicating root synthesis of nornicotine independent of nicotine. The leaf nornicotine also contained a greater excess of $N^{15}$ than did leaf nicotine, indicating translocation of nornicotine to the leaf.

4. The results here presented may be explained by an hypothesis that alkaloid synthesis is a stepwise process and the distinctive property of the root in this process is the synthesis of some alkaloid precursor(s).

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# EMBRYO DORMANCY OF PINUS JEFFREYI MURR. SEED AS AFFECTED BY TEMPERATURE, WATER UPTAKE, STRATIFICATION, AND SEED COAT [1,2]

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Almost three hundred years ago the English silviculturist, John Evlyn (7), recognized the general problem of forest tree seed dormancy. He did not discuss dormancy as such, but he did report that fall or early winter sowing of acorns, beech-nuts, haws and holly berries was a better practice than spring sowing. He went on to state that ". . . seeds may be prepared for the Vernal by being barrell'd or potted up in moist sand or earth stratum during the winter at the expiration whereof you will find them sprouted and being committed to the earth as apt to take as if they had been sown with the most early . . . and will not be much concern'd with the increasing heat of the season, as such as being crude, and unfermented are newly sown in the beginning of the Spring. . . ." Thus, here is perhaps the first published recommendation for artificial stratification of dormant tree seed. It is obvious he did not know why it was effective—as a matter of fact, we do not know why today, even though we have recognized some of the associated biochemical changes (6, 8, 11, 12, 14).

Since the turn of the century numerous studies on seed dormancy have been reported; see reviews by Miller (11), Baldwin (1), Crocker (4), and Crocker and Barton (5). Specific reference to pine seed indicates that the dormancy mechanism of that seed is not entirely clear. For example to overcome dormancy, at least five different pregermination treatments have been recommended. They are: soaking the seeds in sulfuric acid (17); soaking the seeds in water at room temperature (9); soaking the seeds in water at low temperature (15, 16); cold storage in wet sand or peat moss (2, 3, 13, 23) and removal of the seed coat and papery membrane (19).

The most widely used method of increasing seed germination is the low-temperature moist storage treatment generally referred to as stratification. During this particular type of pretreatment chemical changes apparently take place in the seed, after which the seed germinates. For example, Eckerson (6) found during stratification of *Crataegus mollis* seeds that the fats decreased while there was a progressive increase in sugars, acidity, catalase activity, peroxidase activity, and water-holding capacity. Similar results are reported by Pack (14) for seeds of *Juniperus virginiana, J. communis* and *J. prostrata*. He found that fats and proteins decreased while sugars, amino acids, catalase activity, and respiration all increased during stratification.

On the other hand Koblet (10) found little change in the character of the food reserve in *Pinus strobus* seeds during stratification. Only a slight increase in reducing sugars, amino acids, and soluble nitrogen was noted. From this he concluded that the low temperature treatment facilitated a faster mobilization of the reserves during germination and ". . . that low temperatures could effectively stimulate the plasma or upset its equilibrium thereby giving an impulse to growth."

The major change Mirov (12) found in stratified *Pinus lambertiana* seeds was an increased saturation of the fats. His data also suggested a build-up of auxin in the endosperm during stratification — a change which Haddock (8) subsequently was unable to detect.

Stratification is required by some but not all species of pine for satisfactory germination. The "Woody Plant Seed Manual" (21) lists thirteen native species of pine as not requiring stratification. Nevertheless, when seeds of many of these thirteen species are stratified they germinate more quickly and at a more uniform rate than when they are not stratified (2, 3, 13, 23). Therefore a difference in degree rather than a difference in kind is indicated in these two groups. The same metabolic pattern might well prevail in both groups of pine while the level of substrate, auxin, co-factors and inhibitors might be different.

The dormancy of a large number of temperate zone seeds that responded favorably to stratification can also be overcome by breaking or removing the seed coat. This has generally been interpreted as indicating non-dormant embryos, and the pines are often placed in this category (4, 5, 11, 23). However, Stone and Duffield (19) have reported that under certain conditions the embryo of *Pinus lambertiana* was at least partially dormant.

An attempt by the author in 1949 to evaluate conflicting reports by Mirov (12), Haddock (8), and Stone (18) regarding auxin change during stratification was only partially successful (unpublished data). Auxin changes did occur during stratification of *Pinus lambertiana* seed, but it varied from one seed lot to the next. It was obvious that "standardized" seed was needed. Therefore, the following year a large amount of seed was collected from one tree, dried to a moisture content of ten percent, and then stored at 5° C in sealed containers. However, even this uniformly treated seed could not be considered "standardized" because germination behavior changed considerably during the first twelve months of storage. Following this discovery it became important to obtain more specific information about germination behavior prior to the continuation of the auxin studies. Consequently a series of germination behavior studies was undertaken.

[1] Received July 3, 1956.

[2] Work carried on under the University of California Agricultural Experiment Station Research Project 1577.

During the ensuing five years a number of experiments were carried out, none of which were very important in themselves. Now, however, an interesting picture can be drawn from the overall data. Not only was the germination followed for *Pinus lambertiana* seed which normally requires stratification but also for *Pinus jeffreyi* which normally does not (21). Relevant parts of this study that deal with *Pinus jeffreyi* are reported here.

## Materials and Methods

The seed used in this study was collected in 1952 from several trees located in northeastern California. After air drying for a week or more the seed was cleaned, sealed in five gallon cans, and shipped to Berkeley. There it was stored at 1° C until used in 1954 and 1955. The moisture content of the seed varied from 10 to 13 %, with an average of 12 %. Cutting tests showed that 4 % of the seeds were empty or otherwise morphologically defective. At the time of this study freshly harvested seed was not available. However, with *Pinus jeffreyi* this does not assume the importance it does with *Pinus lambertiana*. Seeds of *Pinus jeffreyi* store well at 1° C for several years without any appreciable reduction in its germination (21).

Germination techniques, sample size, and the selection of appropriate germination temperatures were based upon preliminary experiments. The germination temperatures selected were 5, 15 and 25° C, and in each case were held to within ± 0.5° C throughout the experiment. The samples were replicated ten times and were of ten seeds each.

As a measure of the effect of the various seed treatments upon germination at the different temperatures, the number of days required to reach 50 % germination was selected. The 50 % germination level was used rather than the 90 % or some other level, because germination was close to its maximum rate at this point and because the total germination of each seed sample used at least reached the 50 % level. For a statistical expression of the significance of the difference of germination at the 50 % germination level, the standard error (S.E.) of the difference of the means was calculated and the t-test applied.

In the water uptake experiments, samples of twenty-five seeds each, replicated five times were used. Although the S.E. of the mean was large the effect of the treatment, e.g., difference in water uptake after removal of the seed coat, was always many times greater and a statistical expression of the significance of the difference was not considered necessary.

Stratification: Two different methods of stratification were used. The first, or standard method consisted of mixing the seed with wet vermiculite, pouring the mixture into pint ice cream cartons, sealing the cartons and then storing them in a cold room at 5° C for three months.

The second method consisted of first soaking the seed for 48 hours in 5° C tap water through which air was continuously bubbled. Then the seed was removed, sealed in large test tubes, without vermiculite or other stratification media, and stored in a cold room at 5° C for three months.

Germination of Whole Seed: These studies were carried out using Petri dishes filled with wet vermiculite. Ten seeds were placed on the bottom of each dish before the vermiculite was added. It was thus possible to observe germination [3] without having to remove the seed from the germinating medium. The Petri dishes were stacked on shelves in the appropriate incubator and inspected daily. From time to time water was added when the surface of the vermiculite appeared dry. Any water not absorbed by the vermiculite was readily observed and drained off after each addition of water. Except for the short periods when measurements were being made, seeds were kept in the dark.

Germination Without the Seed Coat: In general the procedure was the same as outlined by Stone and Duffield (19). Seeds were surface sterilized by soaking for five minutes in bromine water, before being opened in a sterile transfer room. Then after the seed coat and papery membrane, derived from the inner portion of the integument, and the remains of the nucellus were removed, the embryo, still surrounded by the endosperm, was placed upon solidified 1 percent agar in 25 × 200 mm screw top culture tubes. These were then placed in the appropriate incubator and germination regularly observed. Again, except for the short periods when measurements were being made seeds were kept in the dark.

Some contamination did occasionally occur. However, since each seed was in a separate culture tube, contamination did not spread and in these studies was never of any consequence.

Water Uptake Measurements: These measurements were made with: (a) whole seed, (b) seed from which the seed coat and papery membrane had been removed, and (c) isolated embryos and endosperm tissue. In some experiments the seed was stratified, in others it was not, and in still others the seed was killed [4] by heating for one-half hour at 100° C in stoppered test tubes.

One of the methods used to follow water uptake was to place the seed in Erlenmeyer flasks half filled with water through which air was bubbled. Then every twenty-four hours samples of the seed were taken out, the seed coat removed and the moisture content of the combined endosperm and embryo tissues determined.

[3] Germination, as used in this paper, refers to the initial appearance of the radicle. Subsequent elongation of both the radicle and the hypocotyl are not in any way reflected in the germination data as is the case when germination is determined by the number of seedlings appearing above the ground. The need for this distinction will be developed in a subsequent paper.

[4] When seed was so treated the embryos, contrary to embryo behavior from untreated seed, did not show any growth activity when excised. Furthermore there was no measurable gas exchange in such seeds even after they had imbibed water.

Another method was used in following the water uptake of isolated embryos and endosperm tissue. The particular tissue was placed on a stack of water-saturated filter papers in the bottom of a Petri dish. Saturation of the paper was maintained by the repeated addition of sterile water and by keeping the cover on at all times except when weighings were being made. Individual seeds were weighed daily on a Roller-Smith balance in a sterile transfer room. Necessarily seeds were removed from the incubators for short periods of time. However, the maximum time any seed was out during any 24-hour period was 5 minutes. The steps associated with sterile removal of the seed coat as outlined earlier were also followed in the water imbibition studies with endosperm and embryos in order to reduce contamination and the attendant complications.

## Results

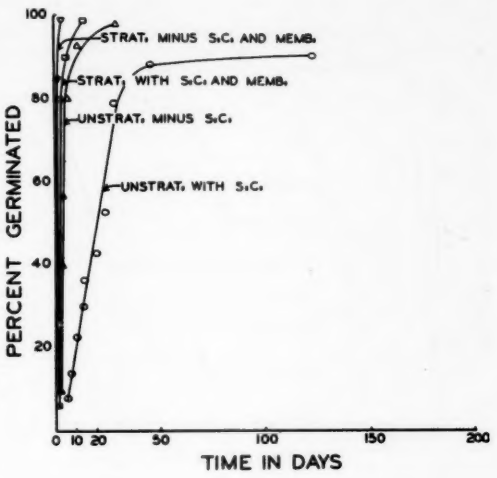
Germination of Stratified and Unstratified Whole Seed: At 25° C, germination of the unstratified seeds reached the 50 % level in 23 days. On the other hand, when stratified, the 50 % level was reached in three days (fig 1).

A ten-degree drop in the germination temperature had a marked effect upon the unstratified seeds, but little effect upon the stratified seeds. Fifty-percent germination of the unstratified seeds required 115 days while the stratified seeds reached the same level in only 6 days (fig 2).

The same general pattern was continued when the germination temperature was reduced to 5° C, although the order of magnitude was different. At this temperature unstratified seeds took 175 days for 50 % to germinate while stratified seed took only 50 days (fig 3). Thus, stratification reduced the germination time at the three temperatures used.

Germination after Removal of the Seed Coat and Papery Membrane: Removal of the seed coats and papery membranes from the unstratified seeds increased the germination rate at all three temperatures. At 25° C the number of days required for 50 % of the seeds to germinate was reduced from 23 to 4; at 15° C it was reduced from 115 to 14; and at 5° C it was reduced from 175 to 95 (figs 1 to 3). Thus here is a pattern similar to that achieved through stratification. However, although the patterns are similar, stratification is more effective in increasing the germination rate than is the mere removal of the seed coats and papery membranes. At 25° C, 50 % of the stratified seeds germinated in 2 days, but it took 4 days when unstratified seed were used and only the seed coats and papery membranes removed; at 15° C it took 6 days and 14 days respectively and at 5° C it took 50 days and 95 days respectively (figs 1 to 3).

When the seeds were stratified and the seed coats and papery membranes were also removed the most rapid germination was attained. Once again the effect of stratification was more evident at 5° C than at 15° C or 25° C. Even at 5° C germination was fairly rapid with 50 % of the germination completed in 10 days. In contrast stratified whole seeds took 50 days, unstratified seeds without seed coats and papery membranes took 95 days, and whole unstratified seeds took 175 days (figs 1 to 3).



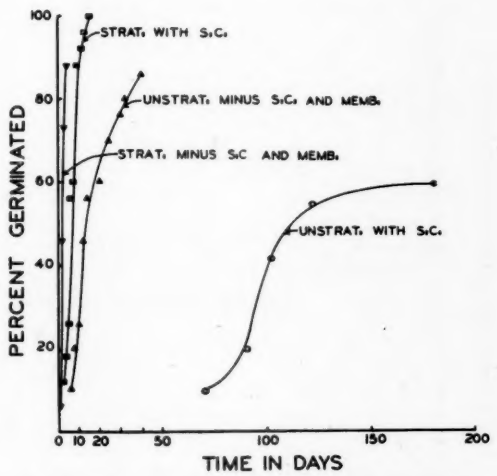
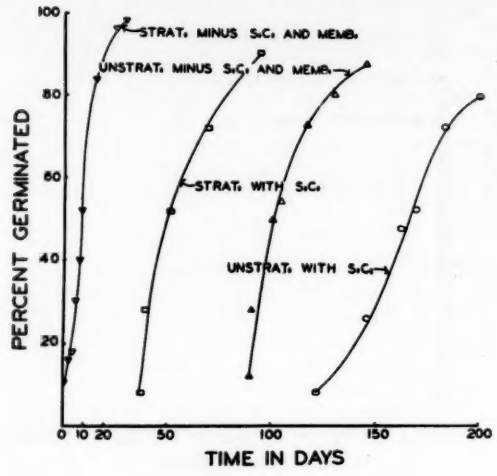




Fig. 1 (*top*). Germination at 25° C of stratified and unstratified seeds, with and without the seed coats and papery membranes. The number of days for 50 % of the seeds to germinate varied significantly (0.95 level) between treatments.

Fig. 2 (*bottom*). Germination at 15° C of stratified and unstratified seeds, with and without the seed coats and papery membranes. The number of days for 50 % of the seeds to germinate varied significantly (0.95 level) between treatments.

These results summarized in figure 4, suggest that stratification and seed coat removal affect germination through their action on two different steps involved in the germination of the seed. They also suggest that embryo dormancy in *Pinus jeffreyi* is a relative con-



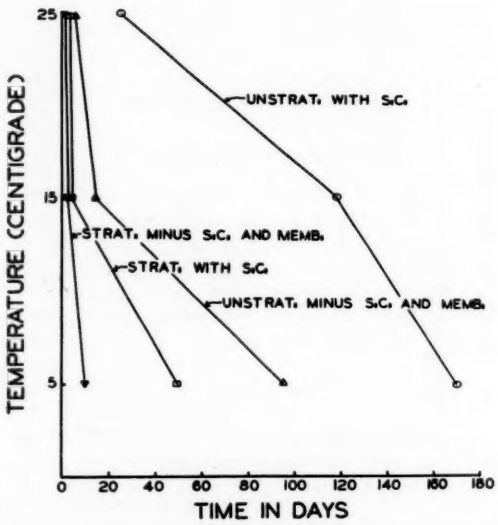



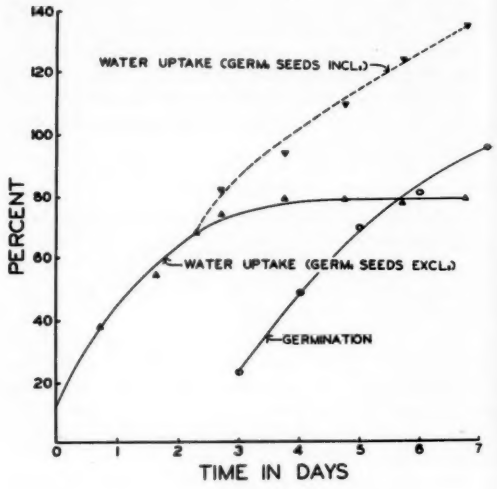

FIG. 3 (*top*). Germination at 5° C of stratified and unstratified seeds, with and without the seed coats and papery membranes. The number of days for 50% of the seeds to germinate varied significantly (0.95 level) between treatments.

FIG. 4 (*bottom*). Average number of days for 50% of the seeds to germinate at 5, 15 and 25° C. Stratified and unstratified seeds, with and without the seed coats and papery membranes are shown. Difference in treatments was significant (0.95 level) at all temperatues.

dition. Further speculation on these points, however, will be deferred until the effect of water uptake on germination is considered.

WATER UPTAKE OF SEED WITHOUT THE SEED COAT AND PAPERY MEMBRANE: At 25° C water uptake is closely associated with germination, with rapid water uptake being followed by rapid germination (fig 5). As a consequence, to the casual observer, it might appear that water uptake per se limits germination. However, such a conclusion is not justified inasmuch as the data do not allow one to specify what is cause and what is effect. This cannot be done unless the two processes can be studied separately.

Fortunately this can be accomplished by simply lowering the germination temperature to 5° C. At this temperature both water uptake and germination



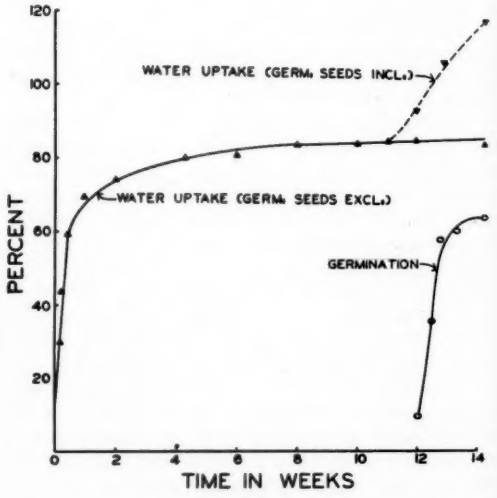




FIG. 5 (*top*). Germination and water uptake (% dry wt) at 25° C of unstratified seeds from which the seed coats and papery membranes were removed. Seeds were germinated on wet filter paper in closed Petri dishes.

FIG. 6 (*bottom*). Germination and water uptake (% dry wt) at 5° C of unstratified seeds from which the seed coats and papery membranes were removed. Seeds were germinated on wet filter paper in closed Petri dishes.

still occur. However, germination does not start for at least three months while water uptake starts immediately (fig 6).

At both 5 and 25° C there is a rapid uptake of water during the first 48 hours. After this initial period the seed continues to take up water but at a much reduced rate (figs 5 and 6).

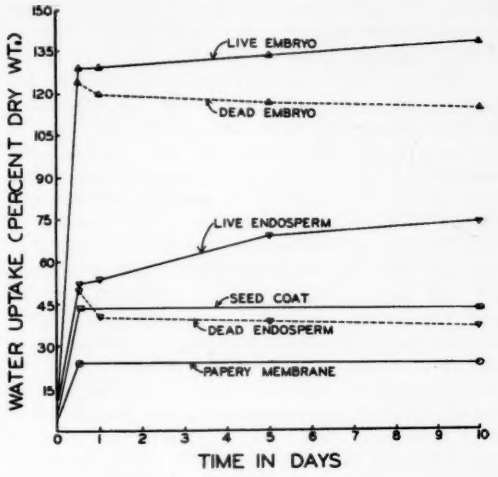
At 25° C the seeds have reached an average moisture content of 68 % after three days, at which point germination begins. In contrast, at 5° C the seeds take 7 days to reach the same average moisture content and germination begins 74 days after that (figs 5 and 6). Thus water uptake alone does not appear to control germination.

Each of the several tissues making up the seed has its own water uptake pattern. Therefore, to obtain a complete picture of water uptake, these individual tissues must be studied both separately and in close association. When each tissue is separated from the seed and placed on water-saturated filter paper water uptake of that particular tissue can be followed by subsequent weighings. When this was done each tissue showed a rapid initial uptake of water (fig 7). This was true for both the dead tissues of the seed consisting of the seed coat and papery membrane as well as for the live tissue consisting of the embryo and endosperm. Even when the endosperm and embryo tissue were killed by heat there was still a rapid uptake of water during the first 24 hours although this in turn was followed by a slight loss in water during the next 24 hours (fig 7).

It would thus appear that the initial water uptake by the seed is the result of imbibitional and osmotic forces. In the dead tissues of the seed only imbibitional forces appear to be involved. In the live tissues made up of the embryo and endosperm osmotic as well as imbibitional forces are indicated, first because the living cell normally contains considerable amounts of osmotically active substances and second because of the loss of water from the dead embryo tissue during the second 24-hour period (fig 7). Such a loss in water can only be explained if one assumes that the semipermeable condition of the cell membrane is destroyed when the tissue is killed by heat. Under such conditions the osmotically active substances would slowly diffuse out of the tissue into the surrounding water and as a consequence there would be a concomitant loss in water from the tissue.

After the rapid initial uptake of water by all the tissues—both dead and alive—the live tissues continue to absorb water at a reduced rate until the seed germinates (figs 5 and 6). This slow uptake is probably associated with a conversion within the cells of osmotically inactive substances such as starch to osmotically active substances such as glucose (5, 6). When the seed germinates there is again rapid uptake of water which is obviously necessary if the newly formed dividing cells of the embryo are to elongate.

At 25° C the water uptake pattern is essentially the same as at 5° C except that there is a telescoping of the time scale. Both curves show a sharp initial rise, followed by a gentle rise, followed finally by another sharp rise when the seed germinates (figs 5 and 6).



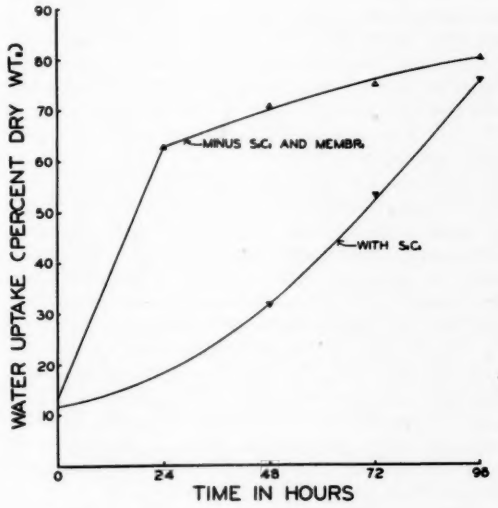




FIG. 7 (*top*). Water uptake (% dry wt) of the individual tissues making up the seed. The various tissues were held on wet filter paper in closed Petri dishes.

FIG. 8 (*bottom*). Water uptake (% dry wt) by intact seeds and seeds from which the seed coats and papery membranes had been removed. Seeds were in tap water at 5° C through which a stream of air was bubbled.

WATER REQUIRED FOR STRATIFICATION: Under normal stratification procedures the seed is free to continue water uptake throughout the period of cold treatment—three months in this case—which it does. This again complicates the interpretation of the relation between germination and water uptake. Is the slow continuous water uptake and resulting high mois-

ture content responsible for the germination behavior of the stratified seed or is this behavior the result of chemical changes within the seed?

Fortunately again, this was easy to determine. Instead of providing a continuous source of water during stratification the seeds were soaked 48 hours in 5° C water through which air was continuously bubbled. Then the seeds were taken out, dried with a paper towel, and sealed in a number of large test tubes. These in turn were placed in incubators at 5, 15, and 25° C for three months. At the end of that time the seeds were removed, planted in wet vermiculite, and then placed in a 15° C incubator. Under similar conditions, seeds stratified by the usual process germinated readily (50 % in five days) while unstratified seeds required at least 118 days (fig 4). Of the treated seeds only those held for 3 months at 5° C had an appreciable germination in less than 60 days. Sixty percent of these seeds germinated in five days thus indicating that the seeds could indeed be stratified by this process. It is therefore apparent that all the necessary uptake of moisture occurred during the first forty-eight hours when the seeds were soaked (fig 8). After that, during the required remaining ninety days, chemical changes essential to rapid germination at 5° C apparently occurred provided the seed was kept at 5° C, but not if it was kept at 15 or 25° C. Thus again moisture per se does not appear to be limiting.

Permeability of the Seed Coat and Papery Membrane to Water: A comparison of water uptake by intact seeds and seeds divested of their seed coats and papery membranes, demonstrated the permeability of these two layers to water. When these seeds were soaked in 5° C water through which air was continuously bubbled, rapid water uptake did occur (fig 8). It is true that water uptake was more rapid when the seed coats and papery membranes had been removed. However, since the seeds still failed to germinate when the lag in water uptake was finally overcome, it would not appear that interference with water uptake per se is the factor limiting germination of *Pinus jeffreyi* seeds.

## Discussion and Conclusions

From the data it would appear that a reasonable working hypothesis for further study of the seed dormancy mechanism in pine can be derived. Basically a two-step process is indicated, such as $S \rightarrow I \rightarrow G$ where S is the substrate, I is one or more intermediate compounds, and G is growth.

First let us examine this hypothesis in light of germination behavior at 5° C (fig 3). At this temperature removal of the seed coat did not in itself promote rapid germination. This only occurred when the seeds had been previously stratified. When the seed coats were removed from the unstratified seeds, germination did not begin for at least ninety days. From then on, however, germination was as rapid as if stratified seed had been used. This would be expected if intermediate compounds I accumulated in the unstratified seed during the initial ninety day period at 5° C. Thus it would appear that at 5° C the seed coat does not interfere with the accumulation of I, but does interfere with its utilization in growth.

Germination of stratified seeds at this low temperature was retarded unless the seed coats were removed. This suggested interference with either water uptake or gas exchange or both. Since the seed coat is permeable to water, uptake of water per se would not appear to be limiting. On the other hand, gas exchange is at least slowed down by the presence of the seed coat. This would tend to reduce the partial pressure of $O_2$ within the seed which in turn could be expected to interfere with certain oxidative reactions necessary for the utilization of material accumulated in the seed during stratification such as the postulated intermediates I.

Thus the proposed reactions $S \rightarrow I \rightarrow G$ in which I slowly accumulates during stratification and in turn is rapidly utilized for growth when the seed coat is removed is compatible with the data presented in figure 3.

Next let us examine the hypothesis in light of germination at 15° C (fig 2). Here again both seed coat removal and previous stratification at 5° C increased the germination rate. However, there was one principal difference. Germination at 5° C, even though the seed coats had been removed, took place only after sufficient time had elapsed for the seeds to become stratified—aproximately ninety days at 5° C for *Pinus jeffreyi*. At 15° C this was not true. Jeffrey pine seeds cannot be "stratified" at this higher temperature. Furthermore, removal of the seed coats from unstratified seeds, where $S \rightarrow I$ would not have been completed, speeded up germination by one-hundred days while removal of the seed coats from stratified seeds where $S \rightarrow I$ would already have been completed germination was only accelerated by five days. Thus it would appear that at this temperature, unlike at 5° C, the seed coat interferes primarily with the $S \rightarrow I$ step.

If at 15° C $S \rightarrow I$ goes on rapidly once the seed coat has been removed, what then can be the explanation for the retarding effect of the seed coat? Once again the partial pressure of $O_2$ in the seed must be considered. It is possible that in the presence of the seed coat and at this higher temperature, with the associated increase in respiratory activity, the partial pressure of $O_2$ in the seed is reduced to a critically low level where S is converted to other compounds C instead of being converted to I. If this occurred there might not be enough I accumulate for subsequent growth and germination, e.g., $S \rightarrow I \rightarrow G$. In effect

$$\searrow C$$

this would involve reactions of the same general type as those associated with the Pasteur effect (20). For additional speculation on the role of oxygen in the germination of dormant and stratified seed, the recent brief review article by Vegis (22) is suggested.

These data indicate the advantages of studying germination at different temperatures. The retarding

effect of the seed coat can be studied at 15° C where it appears to interfere with the accumulation of intermediates. On the other hand at 5° C the effect of the seed coat on the utilization of the intermediates for growth can be studied. Also at 5° C the accumulation of intermediate I and its chemical identification can be most profitably studied.

Thus we have the basis from studies here reported upon for proposing a working hypothesis and also the basis for investigating its validity.

## Summary

1. At 25 and 15° C removal of the seed coats and the papery membranes from unstratified *Pinus jeffreyi* seeds allowed rapid germination while at 5° C it did not. However, when the seed coats and papery membranes were removed from stratified seeds, germination was rapid at 5 as well as at 15 and 25° C. This was interpreted as indicating a condition of "relative" embryo dormancy which was overcome by stratification.

2. The seed coat and papery membrane surrounding the endosperm were found to be permeable to water. Furthermore, "stratification" was accomplished with only that amount of water absorbed by the seed during its first 48 hours in contact with water. Thus water uptake per se did not appear to limit germination.

3. Water uptake and germination were readily separable at 5 but not at 25° C.

4. The data suggested a convenient working hypothesis for the continued study of the seed dormancy mechanism in pine. In effect a two step process was indicated such as S → I → G where S is the substrate, I is one or more intermediate compounds, and G is growth.

5. The effect of the seed coat on germination at 15 and 25° C suggests reactions of the same general type as those associated with the Pasteur effect.

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# METABOLIC PROCESSES IN CYTOPLASMIC PARTICLES OF THE AVOCADO FRUIT. III. THE OPERATION OF THE TRICARBOXYLIC ACID CYCLE[1]

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The evidence for the operation of the tricarboxylic acid cycle in the metabolism of plant mitochondria has been based mostly on studies of oxidative activity. The oxidation of di- and tricarboxylic acids of the cycle was demonstrated in a variety of tissues, such as etiolated seedlings of mung bean (22), pea (10, 31), Avena (32), and Black Valentine bean (4), lupin cotyledons (7), castor-bean endosperm (5, 33), pea internodes (26), cauliflower (15, 16, 17) and broccoli (18) buds, Arum spadix (13, 14), tubers of white potato (28) and of sweet potato (2, 19), avocado fruit (6, 23), spinach leaves (24) and green as well as etiolated pea seedlings and leaves (29, 30). In several of these studies the oxidation rates of acids other than citrate, $\alpha$-ketoglutarate, succinate or malate were also followed (5, 10, 22, 29, 30, 32). The metabolism of pyruvate by the mediation of the cycle was the subject of investigations dealing with young and rapidly growing tissues (4, 5, 7, 10, 22, 32), and to a lesser extent with mature and stable material (2, 6, 19).

Oxidative studies alone do not furnish sufficient proof for the cycling nature and the sequence of the individual reactions. A better insight may be gained by studying the formation of reaction products along with oxidations. This was done only to a limited extent with plant materials. Davies (10) adopted this procedure for a particulate fraction from pea hypocotyl. James and Elliott (14) also cited some chromatographic evidence for mitochondria from Arum spadix.

The objective of this study was to investigate the operation of the cycle in avocado mitochondria by following rates of oxidation of acids and formation of products in the presence and absence of inhibitors.

## MATERIALS AND METHODS

Avocado particles were obtained in a manner similar to that described in the first paper of this series (6). Fruits of the Fuerte variety were peeled and 150 gm of the grated tissue blended in a Waring blendor with 300 ml of a solution 0.25 M in sucrose and 0.05 M in phosphate for 1 minute at 47 volts. The brei was centrifuged at $500 \times g$ for 5 minutes to remove unbroken cells and large fragments. The resultant supernatant was centrifuged at $17{,}000 \times g$ for 15 minutes, and the final pellet suspended in 8.0 ml of the sucrose phosphate solution. This enzyme preparation contained approximately 1.1 to 1.5 mg nitrogen per 0.5 ml of suspension.

All operations preceding the measurement of activity were performed in a cold room maintained at 0 to 2° C. The manometric measurements were carried out at 20° C using conventional Warburg apparatus. The rates of oxidation are reported on an hourly basis since they were linear with time from the outset. The oxidation of fumarate formed an exception, and is treated separately below. All the results were repeated at least once, and the values given are those of a representative experiment.

Paper chromatograms were run following the procedure of Lugg and Overell (20) or its modification (8). The solvents employed were, accordingly, butanol-water (v/v)-formic acid (4 M), or pentanol-water (v/v)-formic acid (5 M). Reaction mixtures were deproteinized by adding to each one drop of 0.5 *N* HCl and placing in boiling water for 1 to 2 minutes. The denatured proteins were centrifuged off and the supernatant applied directly to the paper. With the method, as used, neither pyruvate nor oxaloacetate shows up on the paper unless present in very high concentrations.

$\alpha$-Ketoglutarate was purchased from California Foundation for Biochemical Research; citrate, succinate, malate and fumarate from Eastman Company; pyruvic acid obtained from Eastman was converted into the sodium salt and recrystallized; adenylates and Co A from Pabst Laboratories; DPN and DPT from Nutritional Biochemicals.

The following abbreviations are used: adenosinemonophosphate—AMP; adenosinetriphosphate—ATP; diphosphopyridine nucleotide—DPN; diphosphothiamine—DPT; coenzyme A—Co A.

## RESULTS

OXIDATION OF KREBS CYCLE ACIDS: As can be seen in table I, the avocado particulate preparation was able to oxidize, at a good rate, all the acids of the cycle which were tried. The variability in the rates between the two experiments shown is about as high as was ever observed among different particulate preparations. It should be noted, however, that a consistent pattern was obtained in every experiment, with succinate giving the highest and fumarate the lowest rates. Commercially purchased isocitric acid was also tested, but in view of the rather large amount of contaminants detected chromatographically, the values obtained are of questionable significance and were thus omitted (1). The sample of cis-aconitate used separated chromatographically into two distinct spots (fig 2). One spot agreed in its $R_f$ value, with that of cis-aconitate, while the other was tentatively identified as trans-aconitate. Since the trans-aconitate spot did not change after an incubation with the particulate preparation, it was assumed that the mitochondria acted on cis-aconitate only.

[1] Received July 17, 1956.

TABLE I

OXIDATION OF KREBS CYCLE ACIDS IN THE PRESENCE OF MALONATE AND ARSENITE

| SUBSTRATE* | EXPT 1 | | EXPT 2 | |
|---|---|---|---|---|
| | CONTROL | 0.01 M MALONATE | CONTROL | 0.001 M ARSENITE |
| | $\mu l\ O_2/vessel \cdot hr$ | | | |
| Citrate | 137 | 80 | 179 | 80 |
| Cis-aconitate | 160 | 122 | 224 | 107 |
| α-Ketoglutarate | 178 | 93 | 247 | −9 |
| Succinate | 367 | 89 | 481 | 362 |
| Fumarate | 110 | 73 | 130 | 24 |
| Malate | 151 | 120 | 259 | 40 |

Complete reaction mixture of controls in micromoles: substrate - 60, phosphate - 30, AMP - 3, magnesium - 3, glucose - 60, DPN - 1.5, DPT - 0.2, Co A - 0.07, in a total of 3.0 ml of 0.25 M sucrose.

Particulate preparation contained approximately 1.0 mg nitrogen per vessel. pH 7.0; 20° C; gas phase: air.

* No oxygen uptake in the absence of substrates.

Fumarate, as contrasted with the other acids, was not oxidized at a constant rate from the outset. Its oxidation exhibited a lag period of at least 20 minutes, before significant oxygen uptake could be observed (fig 1). This lag might correspond to the time required for the formation of a sufficient concentration of malate, which is the actual oxidizable substrate. The oxidation rates of fumarate given in the literature were generally low (22, 32) with the exception of the recent work by Beevers and Walker (5) in which the addition of DPN, ATP and Co A resulted in a rate similar to that obtained with other acids.

The unusual pattern of oxaloacetate oxidation by the avocado particles has posed some new problems and is treated in a separate publication of this series (3).

COFACTOR REQUIREMENTS FOR THE OXIDATION OF KREBS CYCLE ACIDS: In order to obtain a washed preparation relatively free of adhering cofactors, the following procedure was followed. The pellet from the high speed centrifugation was resuspended with the use of a close fitting lucite homogenizer in 30 ml of the sucrose-phosphate solution. The suspension was then diluted to 180 ml with sucrose-phosphate and centrifuged again at high speed for 15 minutes. The resultant pellet was suspended in 8.0 ml of sucrose-phosphate, as in the usual preparation. The experiments reported in tables II, III, IV, and V were performed with this washed preparation. In table II single factors were omitted from the complete reaction mixture, while in table III the omission was cumulative. It is evident from both tables that magnesium was required in all cases, while the omission of AMP caused a reduction in the oxidation rates of citrate and α-ketoglutarate. In general, the requirements for the malic oxidation system were similar to those of the other acids. In no case was there a need for DPN, which presumably is particle bound.

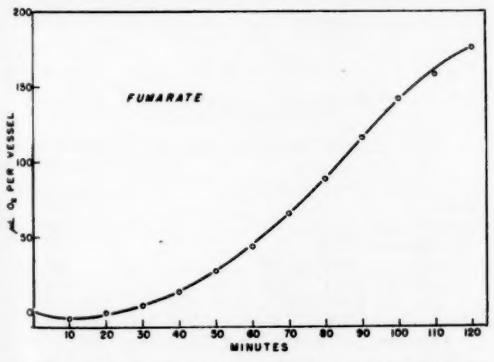


FIG. 1. Oxidation of fumarate by cytoplasmic particles of the avocado fruit. Conditions same as in table I.

TABLE II

COFACTOR REQUIREMENTS FOR THE OXIDATION OF KREBS CYCLE ACIDS (SINGLE FACTOR OMISSION)

| | CITRATE | α-KETOGLUTARATE | SUCCINATE |
|---|---|---|---|
| | $\mu l\ O_2/vessel \cdot hr$ | | |
| Complete | 225 | 248 | 226 |
| Omit DPT | 189 | ... | ... |
| " DPN | 223 | 201 | 236 |
| " glucose | 178 | 211 | 237 |
| " magnesium | 132 | 89 | 153 |
| " AMP | 152 | 109 | 215 |

Complete reaction mixture same as in table I.

DETECTION OF REACTION PRODUCTS: The end products of oxidation for each of the acids were determined two hours after the addition of the particulate suspension to the reaction mixture. The chromatographic patterns at the end of this period were compared with the respective patterns at 0 time, that is immediately after the addition of the enzyme suspension to the reaction mixture. A diagramatic representation of these patterns, based on the actual chromatograms, is shown in figure 2.

TABLE III

COFACTOR REQUIREMENTS FOR THE OXIDATION OF KREBS CYCLE ACIDS (CUMULATIVE OMISSION)

| | CITRATE | α-KETOGLUTARATE | SUCCINATE* |
|---|---|---|---|
| | $\mu l\ O_2/vessel \cdot hr$ | | |
| Complete | 265 | 223 | 253 |
| Omit DPT | 240 | 215 | ... |
| " DPT, DPN | 234 | 226 | 210 |
| " DPT, DPN, glucose | 214 | 178 | 246 |
| " DPT, DPN, glucose, Mg | 112 | 60 | ... |
| " DPT, DPN, glucose, Mg, AMP | 105 | 24 | 141 |

Complete reaction mixture same as in table I.

* For succinate oxidation only DPN, glucose, Mg, and AMP were tested.

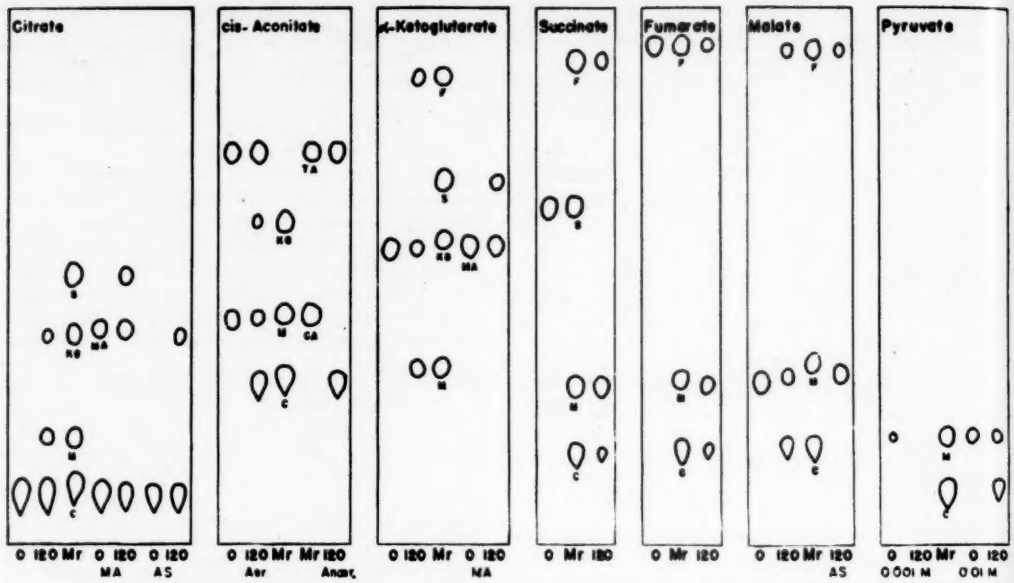


FIG. 2. Diagram of chromatographic separations of reaction products in the oxidation of Krebs cycle acids by cytoplasmic particles of the avocado fruit. Designations: Markers, Mr; citrate, C; malate, M; α-ketoglutarate, KG; succinate, S; cis-aconitate, CA; trans-aconitate, TA; fumarate, F; malonate, MA; arsenite, AS; aerobic conditions, Aer; anaerobic conditions, Anaer.

Concentrations under chromatogram for pyruvate refer to the concentration of malate used as a sparker acid.

It can be seen that after 120 minutes of incubation citrate was converted into malate and α-ketoglutarate; cis aconitate into citrate, α-ketoglutarate and possibly malate; anaerobically cis aconitate was converted to citrate only; aerobically α-ketoglutarate was converted into malate and fumarate; succinate into malate, fumarate and citrate; fumarate into malate and citrate; and malate into fumarate and citrate. All these results are in complete agreement with the operation of the tricarboxylic acid cycle.

EFFECT OF INHIBITORS: The addition of inhibitors to the reaction mixtures changed the pattern of products observed as well as the rate of oxygen uptake. The two inhibitors utilized in this study were malonate and arsenite. Malonate is thought to block chiefly the conversion of succinate to fumarate, while arsenite is considered as a potent inhibitor of pyruvate and α-ketoglutarate oxidations. Since it has been shown that dimercapto systems are much more sensitive to arsenite than single SH groupings, the effect of arsenite on the two oxidase systems has been interpreted as indicating the paritcipation of lipoic acid in one of the intermediate steps (12).

As can be seen in table I and figure 2, our results are in complete agreement with the postulated sites of action of these inhibitors. Table I illustrates that malonate affected succinate oxidation most markedly. Chromatographically (fig 2) no malate could be ob-

TABLE IV

COFACTOR REQUIREMENTS FOR PYRUVATE OXIDATION (SINGLE FACTOR OMISSION)

| | μL $O_2$/MG N · HR |
|---|---|
| Complete | 266 |
| Omit glucose | 211 |
| " DPT | 247 |
| " DPN | 193 |
| " CoA | 209 |
| " AMP | 224 |
| " Mg | 138 |
| " Malate | 18 |
| " Pyruvate | 36 |

Complete reaction mixture same as in table I, plus 6 micromoles of malate.

TABLE V

COFACTOR REQUIREMENTS FOR PYRUVATE OXIDATION (CUMULATIVE OMISSION)

| | μL $O_2$/MG N · HR |
|---|---|
| Complete | 334 |
| Omit glucose | 299 |
| " glucose, DPT | 244 |
| " glucose, DPT, DPN | 206 |
| " glucose, DPT, DPN, CoA | 168 |
| " glucose, DPT, DPN, CoA, AMP | 155 |
| " glucose, DPT, DPN, CoA, AMP, Mg | 116 |
| " glucose, DPT, DPN, CoA, AMP, Mg, Malate | −19 |

Complete reaction mixture same as in table I, plus 6 micromoles of malate.

served as a product of citrate or α-ketoglutarate oxidations in the presence of malonate. Instead, a spot for succinate was clearly visible in both cases. In the case of arsenite, table I illustrates the marked effect of the inhibitor on α-ketoglutarate oxidation. Even at 0.0001 M α-ketoglutarate oxidation was inhibited to the extent of 75 %. When arsenite was added to a reaction mixture containing citrate, no spot corresponding to malate could be observed. The only detectable product was α-ketoglutarate. The effect of arsenite on pyruvate oxidation will be discussed in the following section.

Pyruvate Oxidation and Condensation: No evidence for the operation of the tricarboxylic acid cycle is satisfactory unless it includes proof for rapid utilization of pyruvate through a synthetic reaction involving a four carbon acid with subsequent formation of citrate. The avocado particles were able to oxidize pyruvate provided certain additions were made to the reaction mixture as shown in tables IV and V. The requirement for a sparker is absolute. Malate was used in the experiments reported here, but oxaloacetate could serve as well (3). The omission of magnesium reduced the rate markedly, while the influence of other factors could be observed only when omitted cumulatively. The combined omission of glucose, DPT, DPN, Co A and AMP reduced the rate by over 50 %, though when omitted singly they did not influence the rate greatly. The effects of the inhibitors on pyruvate oxidation are shown in table VI. Arsenite inhibited the reaction virtually completely at 0.001 M, and to the extent of 78 % at 0.0001 M. Malonate caused a 75 % inhibition at 0.01 M. The latter inhibition can be attributed to interference with the formation of the condensing partner, while arsenite presumably affects the lipoic acid step in pyruvate oxidation.

The products of oxidation of 0.02 M pyruvate sparked by 0.001 M malate could not be detected chromatographically (fig 2). Presumably, pyruvate was completely oxidized through the cycle, with none of the acids accumulating in a large enough concentration to appear on the paper. This hypothesis was strengthened by the fact that citrate could be easily detected when the sparker concentration was increased to 0.01 M (fig 2). The addition of malonate (not shown in the diagram) brought out a spot for succinate and a somewhat fainter fumarate spot. In the presence of arsenite no distinction could be made between the 0 time and the 120 minute patterns. However, when arsenite was added to a reaction mixture oxidizing malate (fig 2), no citrate could be observed as a product of the reaction. Thus, the citrate production by malate oxidation, in the absence of arsenite, proceeds most probably, through a decarboxylation to pyruvate followed by the condensation process.

Table VI

Inhibitor Effects on Pyruvate Oxidation

| Inhibitor conc | Arsenite | Malonate |
|---|---|---|
| *moles/l* | *μl $O_2$/vessel · hr* | |
| 0.0 | 223 | 140 |
| $1 \times 10^{-4}$ | 49 | ... |
| $5 \times 10^{-4}$ | 24 | ... |
| $1 \times 10^{-3}$ | 14 | ... |
| $3 \times 10^{-3}$ | − 8 | ... |
| $1 \times 10^{-2}$ | −14 | 36 |

Reaction mixture in control: same as in table I, plus 6 micromoles of malate.

## Discussion

The avocado fruit is, to the best of our knowledge, the only ovarian tissue from which biochemically active cytoplasmic particles were obtained thus far. Attempts were made by Pearson and Robertson (25) to isolate mitochondria from the apple, but their preparations oxidized succinate at a low rate and did not respond to any of the other acids tested. On the other hand, the particles of the avocado were found to carry on both oxidation and phosphorylation (6), to be affected by the uncoupling action of dinitrophenol at certain stages of ripening (23), and to be able to reduce glutathione at the expense of the oxidation of some acids of the tricarboxylic acid cycle (34).

The mitochondrial complex of the avocado appears to be a well integrated entity which has low cofactor requirements. The negative response to exogenous cytochrome c (6) has been explained by the presence of this substance as an integral component of the particle (9). The response to adenylate and magnesium in the α-ketoglutarate oxidation and to magnesium only for the succinoxidase system was shown previously (6) and confirmed here. In this study oxidation rates were compared when one factor was omitted at a time and when the omission was cumulative. With either method the oxidation of citrate, α-ketoglutarate or succinate did not require any exogenous glucose, DPN or DPT. In general, the cofactor requirements for the avocado particles were similar to those of most plant tissues, with the exception of the castor bean endosperm (5) and the sweet potato tuber (19). The mitochondrial oxidations of the latter tissues were stimulated by DPN, DPT and Co A. It seems that neither differences in preparative procedure, nor in rates of oxidation could account for the discrepancy.

The cofactor requirements for pyruvate oxidation by particulate enzymes of the avocado were essentially the same as those for the other acids, except for the need for catalytic quantities of a sparker. An absolute requirement for a sparker acid was shown also for mungbean seedlings (22), cauliflower buds (17), lupin cotyledons (7), Arum spadix (13), oat seedlings (32), castor bean endosperm (5), Black Valentine bean (4) and sweet potato tuber (19). In several other cases malate was added to a reaction mixture containing pyruvate, but was not shown conclusively to be necessary. Malate has been used in nearly all studies, though the obvious choice for a condensing partner with pyruvate is oxaloacetate.

The difficulty experienced with oxaloacetate was probably the result of a lag in oxygen uptake observed when it was applied as a sparker. This lag was reported for lupin cotyledon (7), Avena seedlings (32), castor bean endosperm and white potato (33) and for the avocado fruit (3). In a study of this phenomenon (3) we concluded that this lag was due to the simultaneous oxidation of pyruvate and reduction of oxaloacetate, resulting in no net oxygen uptake.

The role of the sparker acid becomes evident when one follows reaction products along with oxidation rates. Davies (10) detected citrate when pyruvate and oxaloacetate were incubated with the mitochondrial preparation from pea seedlings. A similar observation was made by Lieberman and Biale (19) when pyruvate and malate were added to sweet potato particles. The chromatograms obtained from avocado mitochondrial oxidations clearly show the formation of citrate from pyruvate and malate in this study, or from pyruvate and oxaloacetate reported elsewhere (3). Citrate was also detected in this study as a product of the oxidation of malate, fumarate, or succinate.

Brummond and Burris (7) established the entry of pyruvate into the cycle by a different procedure than most workers. They incubated mitochondria from cotyledons of etiolated white lupine seedlings with pyruvate-2-$C^{14}$ and unlabeled malate. At the end of the incubation period the molar specific activities of citrate and pyruvate were essentially the same, indicating that citrate arose from pyruvate directly. In addition, they isolated all the other acids of the cycle (using added carrier acid) and showed significant incorporation of the label in each. They concluded that the tricarboxylic acid cycle operates in white lupine mitochondria but "whether it is functional in mature tissue remains to be established." We submit that the evidence obtained since and presented here coupled with observations of others, strongly suggests the operation of the cycle in mature tissues of several species.

It is probably correct to say that not all the reactions essential for the functioning of the cycle have been sufficiently studied. This is particularly true with respect to the rather involved condensation process. Davies (11) stated that fluoroacetate failed to cause accumulation of citrate, though in non-poisoned systems citrate was observed chromatographically. Walker and Beevers (33) demonstrated the formation of acetyl phosphate from pyruvate, when trans-acetylase was added to the plant mitochondrial preparation. This strongly suggested acetyl Co A as an intermediate in pyruvate oxidation. The wide occurrence of Co A in higher plants (27) is suggestive, although by no means conclusive, of its role in pyruvate metabolism. It was also shown that solubilized preparations from a variety of plant tissues are able to form acetyl Co A from acetate, Co A and ATP (21). The participation of DPT in pyruvate oxidation was indicated by the marked increases in oxygen uptake observed upon its addition in some tissues (33, 19). The high sensitivity of the pyruvate and $\alpha$-ketoglutarate oxidation systems to arsenite, demonstrated in this study, suggests that lipoic acid may also be involved, as shown previously for animal and bacterial preparations (12).

The evidence for the entry of pyruvate into the cycle has to be coupled with proof for its oxidation through the catalytic action of the acids. This can be done by the use of several techniques. One can show the ability of all the acids to serve as sparkers for pyruvate oxidation (32, 33). The technique used by Brummond and Burris (7) and discussed above provides another way. However, neither of these methods indicates the sequence in which the reactions proceed. By following the products of the reactions and the effect of inhibitors on them this additional information can be secured. In this manner, we obtained evidence regarding the acids participating in the cycle and the order in which these reactions are arranged, which is in complete agreement with the tricarboxylic acid cycle.

In conclusion, it could be said that the results of all investigations regarding the operation of the tricarboxylic acid cycle in plant tissues provide strong evidence for regarding it as a major pathway of oxidation in particles isolated from a variety of higher plant tissues.

## Summary

This paper is concerned with the operation of the tricarboxylic acid cycle in particles obtained by differential centrifugation from avocado fruits. With the aid of manometric and paper chromatographic techniques the oxidation and appearance of the expected products was shown when each of the acids was incubated with the enzyme preparation.

The effect of malonate on the inhibition of oxidation and appearance of succinate as product was shown for several acids. The effect of arsenite on the oxidation of the acids and product formation was found to agree with its action on animal mitochondria-inhibiting pyruvate and $\alpha$-ketoglutarate oxidation.

Cofactor requirements were studied for the oxidation of pyruvate, citrate, $\alpha$-ketoglutarate, succinate and malate. Magnesium ions were required in all cases, while the requirement for the other cofactors varied with the substrate used. A sparker quantity of malate was found to be an absolute necessity before any pyruvate oxidation proceeded.

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# ASPECTS OF PHOSPHORUS METABOLISM IN THE COTTON PLANT[1]

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The results presented in this paper were developed from comparisons of the growth and phosphorus fractions in cotton plants grown in high- and low-P solutions: (a) Plants with bolls in sand cultures were sampled at 35 and 87 days to measure growth and to obtain data on carbohydrate and nitrogen fractions, alcohol-soluble and total phosphorus, and mineral constituents; and (b) plants without branches or bolls in water cultures were sampled at 34 and 61 days for the phosphorus fractions: inorganic, soluble organic, nucleic acid, protein, and total organic. Phosphorus-fractionation data are reported, also, for 10-day-old ovules and mature seed kernels.

During grain development the highly determinate cereals draw heavily on phosphorus previously stored in the stems and leaves. As shown in the succeeding paper (7), the cotton plant when growing upon a fertile soil develops its reproductive tissues largely on the basis of a continued uptake of phosphorus with only minor exhaustion of the phosphorus of the leaves and stems. With the withdrawal of the phosphorus supply, however, the P concentrations in the vegetative tissues are found in this paper to be only a fraction of that when the P supply is ample. This latter finding is in keeping with results of Arnon and Hoagland (2), who grew tomatoes for five weeks in complete nutrient solutions and then transferred half of the plants to minus-P solutions. The tomato plants made only one-fifth as much growth and the young leaves at the end contained only a fifth as much phosphorus (the old leaves only one-fifteenth as much) as the corresponding leaves on their control solutions. Deflowering approximately doubled the growth of the low-P plants. Arnon, Stout, and Sipos (3) found that fruit of tomatoes had a "physiological priority" in the accumulation of radio-active phosphorus. Pirson in a recent review (20) has stressed the paucity of evidence on the finer changes which take place in the various phosphorus fractions with the withdrawal of substrate phosphorus; the present paper places special emphasis upon this phase of the phosphorus relations of the cotton plant.

## ANALYTICAL METHODS

Triplicate 1.5 gm samples of fresh leaf tissue (60 to 70 1-cm leaf punches) were fractionated into inorganic, soluble organic, nucleic acid, and protein phosphorus by the procedures of Juni et al (15) as modified by Klein (13). Lipid phosphorus, determined as above, is reported for ovules and seed kernels, but it is included in the nucleic-acid fraction in the instance of leaves since preliminary trials had shown the lipid-P to comprise less than 0.001 % of the dry weight. When measured directly, total phosphorus was determined after wet-ashing with concentrated $H_2SO_4$ by the method of Fiske and Subbarow (7). When checked directly, the sums of the various fractions have agreed well with values for total phosphorus. The mineral analyses were by Official Methods and the carbohydrate determinations by the methods used heretofore in this laboratory.

## METHODS AND PROCEDURES

Hoagland-type nutrient solutions were used in all the experiments. The Hoagland solutions, plus 1 Mm/l of NaCl, the usual microelements, and iron as sequestrene NaFe, were made up with analytical grade chemicals in deionized water. The low-P solutions were the same as the high-P solutions except that 1 Mm/l of KCl was substituted for the Mm/l of $KH_2PO_4$ (31 ppm of P) of Hoagland's solution. Recurrent analyses of the original low-P solutions for phosphorus derived from chemical impurities showed concentrations that varied between 1 and 2.0 ppm in the several experiments as conducted over a period of two years.

The sand cultures were 24-inch-tall, 4-gallon stone jars filled with coarse quartz sand that had been leached after use in other experiments, but because of the previous use, it may have contained some slowly soluble phosphate. The jars had drainage outlets fitted with a glass tube that extended upward for 8 inches to provide for extra storage of solution in the bottom of the sand. As wetted to overflow the tube, each jar retained about 5 liters of solution. New solution, or deionized water, was added daily.

The water cultures were 20-liter stone jars with wooden covers. The plants were held in place in holes in the covers with cotton packing. The cultures were continuously aerated through gas-diffuser stones.

The water-culture solutions were changed at irregular intervals and extra P was added to the low-P solutions in order to support some growth of the plants. Between solution changes, the volumes were maintained by adding deionized water. The proportions of Ca, Mg, K, N, P, and S in Hoagland's solution correspond well (6) with the proportions found in cotton plants grown on fertile field soils. In renewing the solutions, this fact was borne in mind and, also, that 20 liters of Hoagland solution should produce a 500-gm cotton plant (fresh weight including the roots) if all nutrients were taken up.

## EXPERIMENTAL DATA

GROWTH AND PHOSPHORUS, CARBOHYDRATE, NITROGEN, AND MINERAL ACCUMULATIONS IN FRUITED SAND-CULTURE PLANTS: By February 12, after planting in the quartz sand on January 18, the low-P

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plants were less than half as large as those in high-P and the petioles of the cotyledons had started to turn red. Phosphorus determinations on the solutions as drawn from the sand at that time showed 24 ppm in the high-P cultures and 0.96 ppm in the low-P cultures. One liter of new solution containing a total of 7.8 ppm of P was then added to all cultures. The first plant samples (12 plants selected from the high-P jars and 22 from the low-P jars) were taken on February 22 (day 35) and one plant was left in each of the 12 jars of each treatment for the final sampling on day 87. When sampled on February 22 the lower leaves of the low-P plants were losing their chlorophyll and abscising. Starting on February 22, 1 liter of nutrient solution containing 7.8 ppm of P was added daily for 21 days to the low-P jars and complete solution to the high-P. Thereafter to the 87th day, 1 liter of the respective normal and low-P (1.4 ppm P) solutions were added on alternate days. The plants were watered with deionized water to produce some drainage on the intervening days.

Much uncertainty always exists as to the actual concentrations of the various constituents of nutrient solutions in sand cultures—unless there is automatic reflushing from, and return drainage to, a reservoir. But from the foregoing data it is believed that the high-P cultures had concentrations around 25 to 30 ppm of P at all times and that the low-P cultures rarely had much less than 1 ppm; for the period from 35 to 56 days the concentrations should have been substantially above 1 ppm.

*Growth:* As shown in table I both at 35 and 87 days, the fresh weights of the plants in the low-P cultures were only one-eighth as great as the corresponding values in high-P cultures. The heights of the low-P plants were reduced by one-half and the numbers of main stem nodes were reduced by 40 % of the high-P values. The relative fruitfulness (bolls per 100 grams of fresh stems and leaves) of the low-P plants was reduced to a quarter of that of the high-P plants. The effects of inadequate phosphorus were thus reflected by sharp curtailments both in growth and in the differentiation of new nodes and by the even greater curtailments in the development of the reproductive tissues—both the fruiting branches and their floral buds and bolls.

The foregoing results, indicating the need for high concentrations of substrate P, are in good accord with an experiment by Mason and Phillis (17) who also grew cotton in sand cultures. Although these investigators do not report on the extent to which their substrate concentrations were depleted, they found a sharp upward rise in growth and P accumulation as the P in their culture solutions was increased from 10 to 40 ppm. That the phosphorus requirements of cotton stands in contrast with that of some other plants grown on nutrient solutions is indicated by Parker's results (19) with soybeans and corn; these plants made maximum growths when on solutions maintained with 0.50 ppm of phosphorus. With sand-culture nutrients allowed to become depleted (12), high initial P concentrations were found to be beneficial to soybeans. In contrast with soybeans and corn, Chapman and Rayner (5) found orange trees to develop marked deficiency symptoms with 1 ppm and unmistakable symptoms when maintained with 4 to 5 ppm of phosphorus.

For the purposes of determining further whether cotton requires higher substrate concentrations of phosphorus in nutrient solutions than do other plants, or whether our results were peculiar to the sand culture technique employed, a short accessory experiment was conducted in water cultures in 20-liter jars with aerated Hoagland-type solutions with high (4 jars) and moderate levels (4 jars) of P. The nutrient solutions were not changed or additional P added during the test. The hydrogen ion concentration was maintained between pH 5.5 and 6.0 by additions of $HNO_3$. The plants, after seed germination, were grown in the water cultures for 25 days. The initial

TABLE I

GROWTH AND FRUITING OF STONEVILLE 2B UPLAND COTTON PLANTS IN SAND CULTURE AS INFLUENCED BY PHOSPHATE SUPPLY

| Phosphate level | Fresh wt | | Main stem | | Fruiting branches | | Buds ** at harvest | Bolls | | Relative fruitfulness † |
|---|---|---|---|---|---|---|---|---|---|---|
| | Entire plant * (gm) | Leaves & stems (gm) | Ht (cm) | Nodes | Mean length (cm) | No. | | Shed | Retained | |
| | | | | | *35 days* | | | | | |
| High | 17.3 | ... | 25 | 7.1 | ... | .. | ... | .. | ... | .. |
| Low | 2.2 | ... | 12 | 4.2 | ... | .. | ... | .. | ... | .. |
| | | | | | *87 days* | | | | | |
| High | 747 | 544 | 143 | 24.3 | 42.1 | 14 | 35.3 | 3.0 | 24.5 | 4.5 |
| Low | 81 | 71 | 71 | 16.5 | 12.3 | 3 | 6.0 | 0.0 | 0.7 | 1.0 |

Values given are the averages per plant. Cotton was planted January 18.
* Includes roots and reproductive tissues.
** With 1 cm or greater bract width.
† No. of developing bolls per 100 gm of fresh leaves and stems.

and final concentrations of P in the solutions and the final fresh weights of leaves plus stems per plant were as follows:

| | P IN SOLUTIONS, PPM | | FRESH WT OF LEAVES AND STEMS, GMS |
|---|---|---|---|
| | INITIAL | FINAL | |
| High-P | 32.0 | 20.0 | 18.7 |
| Moderate-P | 7.6 | 2.6 | 1.7 |

In this test, where there could be no question regarding the phosphate concentrations at the root surfaces, the results are even more difficult to understand than those of the foregoing sand-culture experiment. With never less than 2.6 ppm of phosphorus in the low-P cultures, which were gently circulated by reason of aeration, growth was reduced to a tenth of that obtained in the high-P cultures which had a minimum of 20 ppm of phosphorus.

Sommer (21) noted the absence of root hairs on cotton plants grown in water cultures and we could find none in ours. She concluded that cotton required higher culture-solution concentrations of P than other plants compared. Root hairs were present in our sand culture experiment, and they are present when seed are germinated in kraft towels. The writers know of no evidence which can be interpreted as indicating that cotton growing on soils require a higher concentration of phosphorus than other plants but it is possible that soil solutions have not been examined with this point in mind. The review by Arnon (1) stresses the usual adequacy of 0.1 ppm of P in displaced soil solutions where equilibriums exist with fixed phosphorus.

*Phosphorus Accumulations:* The phosphorus designated "alcohol soluble" in table II is that in aliquots of the 80 % alcoholic extracts for the sugar determinations; the tissues were killed in hot alcohol. It was assumed when the analyses of the alcoholic extracts were undertaken that the phosphorus found in such extracts would be largely that of the phosphorylated sugars, or their intermediates.

In terms of all tissues, the high-P plants averaged five times more total phosphorus than did the low and, as is usual, the concentrations in the leaves exceeded those of the stems. Only a small part of the total phosphorus was present as alcohol-soluble organic phosphorus. In the leaves of the high-P plants, the average ratio of total to alcohol-soluble phosphorus was 19 to 1 and in the low-P plants the ratio was 13 to 1.

*Carbohydrate Accumulations:* In terms of all tissues and the two sampling dates, the average concentration of both reducing sugars and starch were almost four times higher in the P-deficient plants than in the high-P plants (table III). Sucrose was variable and only in the instance of one sample was there appreciably more sucrose in the deficient than the

TABLE II

ALCOHOL-SOLUBLE AND TOTAL PHOSPHORUS AT PREFRUITING STAGES OF COTTON PLANTS CULTURED IN SAND

| TISSUES | AV DRY WTS, GM | | % PHOSPHORUS | | | |
|---|---|---|---|---|---|---|
| | | | ALCOHOL SOLUBLE | | TOTAL | |
| | HIGH-P | LOW-P | HIGH-P | LOW-P | HIGH-P | LOW-P |
| | | | *35 days* | | | |
| Leaf blades | 1.16 | 0.24 | 0.016 | 0.016 | 0.605 | 0.059 |
| Other * | 0.70 | 0.15 | 0.028 | 0.005 | 0.327 | 0.036 |
| | | | *87 days* | | | |
| Leaf blades | | | | | | |
| Upper | 7.1 | 3.5 | 0.005 | 0.024 | 1.474 | 0.318 |
| Lower | 7.5 | 2.2 | 0.046 | 0.014 | 0.902 | 0.253 |
| Main stems | | | | | | |
| Upper | 10.5 | 3.4 | 0.029 | 0.006 | 0.454 | 0.106 |
| Lower | 9.5 | 2.8 | 0.032 | 0.007 | 0.425 | 0.098 |

Data expressed on dry wt per plant. Planted January 18.
* Included stems, petioles and roots.

TABLE III

CARBOHYDRATES AS INFLUENCED BY PHOSPHORUS SUPPLY

| TISSUES | REDUCING SUGARS | | SUCROSE | | STARCH | | HEMICELLULOSE | |
|---|---|---|---|---|---|---|---|---|
| | HIGH-P | LOW-P | HIGH-P | LOW-P | HIGH-P | LOW-P | HIGH-P | LOW-P |
| | | | | *35 days* | | | | |
| Leaf blades | 0.21 | 0.65 | 0.16 | 0.18 | 3.41 | 5.21 | 2.89 | 5.60 |
| Other * | 0.36 | 1.24 | 0.10 | 3.33 | 0.99 | 8.14 | 11.82 | 12.86 |
| | | | | *87 days* | | | | |
| Leaf blades | | | | | | | | |
| Upper | 1.47 | 2.79 | 1.85 | 1.24 | 9.52 | 12.31 | 5.28 | 5.20 |
| Lower | 0.45 | 1.33 | 1.51 | 1.51 | 3.82 | 10.76 | 3.27 | 4.37 |
| Main stems | | | | | | | | |
| Upper | 1.09 | 3.61 | 4.99 | 5.85 | 1.68 | 6.08 | 15.54 | 14.77 |
| Lower | 0.14 | 1.33 | 3.25 | 3.67 | 2.57 | 13.37 | 14.57 | 12.98 |

Data as percentages of dry wt. Planted January 18.
* Included roots.

high-P plants. Hemicellulose was not markedly or consistently influenced.

In the presence of phosphorylase (11), factors causing a lowering of inorganic phosphate (4) should tend to increase starch synthesis:

$$\text{Glucose-1-phosphate} \rightleftharpoons \text{Starch} + H_3PO_4$$

The present data accord well with theory in this regard, but an examination of the literature indicates that low-P plants do not always accumulate extra starch. Hall (10) working with four phosphate-supply levels found increased starch in the leaves of tomato plants at intermediate levels of P-supply but not at his lowest and highest levels. MacGillivray (16), also with tomatoes, obtained data corresponding with our data as regards reducing sugars, sucrose and hemicellulose but starch was decreased with low-P rather than increased; the same trends are shown in the data on tomatoes by Janssen et al (14). Steinberg's (22) review also indicates a substantial variability in the carbohydrate responses to low phosphorus in other plants.

The fact that uniform effects from phosphate deficiency have not been found on carbohydrate accumulations among different plants provides support for the view that the curtailment of growth associated with phosphate deficiency is in great part the result of photosynthetic inhibition.

For the complete phosphorylation of reducing sugars, it is required that one mole of phosphorus be present for each mole of glucose and two moles of P for each mole fructose. If it is assumed that the concentrations of glucose and fructose are equal, then there must be one gram of alcohol-soluble phosphorus for each 4.35 grams of reducing sugar. The ratios of the alcohol-soluble P (table II) to that required for complete phosphorylation of the reducing sugars (table III) are as follows:

| Sample | Ratios of P found to P required | |
|---|---|---|
| | High P | Low P |
| 35 days: | | |
| Blades | 1.27 | 0.11 |
| Other | 0.34 | 0.03 |
| 87 days: | | |
| Blades, upper | 0.16 | 0.04 |
| " , lower | 0.44 | 0.06 |
| Stems, upper | 0.16 | 0.03 |
| " , lower | 1.00 | 0.02 |

In this tabulation the ratios of P found in the alcoholic extracts to the P required for complete phosphorylation are far lower in the low than in the high-P plants. The average of all samples indicate around 50 % of phosphorylation under high-P and about 5 % under low-P. In the lower stems of the high-P plants at 87 days complete phosphorylation is indicated but this value has little significance since the tissues contained only 0.14 % of reducing sugar. Evidence of complete phosphorylation is found only in the high-P young leaves at day 35. It seems possible, or even probable, that the alcohol could have extracted some phosphorus that was not tied to sugar; if such an assumption is made, the ratios shown are maximum values and the conclusion is further reinforced that in cotton tissues, the phosphorylation of the reducing sugars is ordinarily low and rarely complete.

*Nitrogenous Accumulations:* The effects of phosphorus deficiency on the accumulation of the various nitrogen fractions (table IV) were not always consistent between tissues and age of plants but there were certain trends. When the data for the stems and leaves of both the 35- and 87-day-old plants are averaged, the percentage of nitrate N was reduced by P deficiency by an average of 18 % and the percentages of protein and total N by 16 and 13 %, respectively. On the other hand, soluble organic N was increased by 14 %. Steinberg (22) has reviewed earlier data in this field. In keeping with the present data, he observed the usual tendency to be toward increased amounts of amino nitrogen and decreases in protein nitrogen; this had been reported for barley, wheat, and tobacco but not for tomatoes. A deficiency of phosphorus should limit the elaboration of amino acids into the more complex phosphorus-containing nucleoproteins. Phosphorus deficiency in the present experiment with cotton reduced, rather than accelerated, nitrogen uptake.

*Mineral Accumulations:* With the phosphorus deficiency there were small but rather consistent decreases in the percentage accumulations of calcium, magnesium, potassium, and sulphur, table V, as well as of nitrogen mentioned above. In the single measurements on the younger leaves, it seems that sodium accumulation may have been increased by the deficiency. There is no evidence of any effect upon the percentage of iron.

Although not explaining the extra accumulation of sodium in the low-P leaves, nor throwing light on questions involved in the functioning of carrier molecules and balances between sodium and other ionic accumulations, it remains logical that the extra ionic accumulations (percentage composition) by the plants on the high-P substrates reflects some respiratory advantage in terms of phosphate-bond energy. Overstreet and Jacobson (18) and Arnon (1) have commented on the involvement of phosphorylated intermediates in inorganic accumulations. It has been suggested that high-energy phosphate bonds, as a part of carrier molecules, are intimately involved in respiratory accumulations of ions against concentration differentials.

Phosphorus Fractions in Leaf Blades of Plants Grown in Water Cultures: The plants used for the phosphorus-fraction measurements were grown without branches or bolls (buds pruned out) on water cultures after transferring them to high- and low-P solutions from the germination sand (no nutrients) on their 14th day. Plants were harvested for analyses on the 34th day after planting and the remaining plants in high-P treatment then divided between high- and low-P solutions for an additional 27 days. During this final period of 27 days both groups

TABLE IV

NITROGEN FRACTIONS AS INFLUENCED BY PHOSPHORUS SUPPLY

| TISSUES | NITRATE | | SOLUBLE ORGANIC | | PROTEIN | | TOTAL | |
|---|---|---|---|---|---|---|---|---|
| | HIGH-P | LOW-P | HIGH-P | LOW-P | HIGH-P | LOW-P | HIGH-P | LOW-P |
| | | | | *35 days* | | | | |
| Leaf blades | 0.35 | 0.37 | 0.11 | 0.08 | 3.96 | 2.63 | 4.42 | 3.08 |
| Other * | 1.26 | 0.56 | 0.09 | 0.09 | 1.23 | 0.78 | 2.58 | 1.43 |
| | | | | *87 days* | | | | |
| Leaf blades | | | | | | | | |
| Upper | 0.24 | 0.20 | 0.41 | 0.42 | 3.23 | 2.84 | 3.88 | 3.46 |
| Lower | 0.14 | 0.23 | 0.60 | 0.65 | 2.57 | 2.85 | 3.31 | 3.73 |
| Main stems | | | | | | | | |
| Upper | 0.11 | 0.32 | 0.24 | 0.38 | 0.59 | 0.63 | 0.94 | 1.33 |
| Lower | 0.12 | 0.11 | 0.23 | 0.31 | 0.43 | 0.40 | 0.78 | 0.82 |

Data as percentages of dry wt. Planted January 18.
* Included roots.

of plants made over 90 % of their total growth. Since so great a part of the total growth was made during this latter period, emphasis is placed in the discussion on the partition of the phosphorus between the several phosphorus fractions in the several tissues rather than on the hydrolysis and transport of the phosphorus accumulated during the early period of growth. The growth of leaves, stems, and roots and their total phosphorus concentrations are presented in table IV and the fractional distributions in table VII.

During the early period (table VI), the high-P plants made about four times as much growth as did the low-P plants and they accumulated (including that supplied by the seed) 27 times the weight of phosphorus. In terms of weighted-mean percentage composition, the high-P plants were seven times as high in phosphorus as the low-P.

Relative to the weights of corresponding leaves, stems, and petioles, the weight of the roots in low-P was almost twice that in the high-P. During the first period, the concentration of total phosphorus in the high-P roots was ten times that of the roots in low-P.

During the first period the high-P plants showed little difference in phosphorus concentration between leaves, stems, and roots. In the low-P treatment, on the other hand, the leaves had a high priority; the leaf concentrations were more than double those of the roots or stems.

At day 61, relative to day 34, there were substantial reductions in the high-P plants in the total phosphorus concentrations found in leaves, and particularly in the stems and roots. Under P-deficiency, the concentrations found in the three respective tissues were almost identical at the two sampling dates. Had the branch buds (and therefore, the reproductive tissues) not been removed, the reductions of P in vegetative organs under high-P supply would doubtless have been greater between the two dates.

Symptoms of phosphorus deficiency were marked in the leaves of the low-P plants (fig 1). Starting early in the growth of the plants, irregular areas in expanding leaves became chlorotic and patches of tissue then dried and turned white. Chapman and Rayner (5) picture similar symptoms for phosphorus-deficient orange leaves. Howell's illustration (12) of

TABLE V

MINERAL ACCUMULATIONS IN COTTON LEAF BLADES (ALL) AS INFLUENCED BY PHOSPHORUS SUPPLY

| ELEMENT | HIGH-P | | LOW-P | |
|---|---|---|---|---|
| | 35 DAYS | 87 DAYS | 35 DAYS | 87 DAYS |
| Calcium | 4.48 | 4.73 | 3.45 | 4.36 |
| Magnesium | 0.63 | 0.79 | 0.52 | 0.65 |
| Potassium | 2.64 | ... | 2.35 | ... |
| Sodium | 0.34 | ... | 0.64 | ... |
| Sulfur | 1.17 | 0.93 | 0.91 | 0.83 |
| Iron | ... | 0.027 | ... | 0.026 |

Data as percentages of dry wt. Planted January 18.

TABLE VI

DRY WEIGHT AND TOTAL PHOSPHORUS OF EMPIRE COTTON PLANTS * GROWN WITHOUT BRANCHES IN WATER CULTURES

| PART | GM DRY WT | | % TOTAL PHOSPHORUS | |
|---|---|---|---|---|
| | HIGH-P | LOW-P | HIGH-P | LOW-P |
| | | *34 days* | | |
| Leaf blades | 1.78 | 0.48 | 1.30 | 0.24 |
| Stems, petioles | 0.89 | 0.10 | 1.22 | 0.12 |
| Roots | 0.83 | 0.32 | 1.44 | 0.13 |
| Totals & wt means | 3.50 | 0.90 | 1.31 | 0.19 |
| | | *61 days* ** | | |
| Leaf blades | 34.8 | 24.6 | 1.00 | 0.40 |
| Stems, petioles | 30.9 | 23.1 | 0.33 | 0.11 |
| Roots | 15.0 | 13.2 | 0.46 | 0.14 |
| Totals & wt means | 80.7 | 60.9 | 0.64 | 0.23 |

* Planted May 21.
** All plants sampled at 61 days were grown on high-P solutions through day 34.

FIG. 1. Symptoms of phosphorus deficiency as developed in cotton leaves during the summer in a warm-to-hot greenhouse.

P-deficient soybean leaves shows the blanching to have occurred only between the veins.

To permit differentiations in the phosphorus-fraction compositions of leaves as influenced by age, as well as by P-supply, a tag was placed on the uppermost unfolded leaf at the time of harvesting the 34-day samples; the 61-day leaves are reported as "old" or "new" according to this age grouping. The 34-day leaves of the first sampling and the "old" leaves at 61 days are from the same positions on the main stems but at the latter date they were 27 days older.

Of the total phosphorus in the leaves of the high-P plants without branches, nearly one-half was inorganic whereas with the low-P supply only a quarter, or less, was inorganic. The weighted-mean total phosphorus concentration was 2.5 times greater in the high-P than in the low-P leaves.

It had been presumed at the start of the experiment that the high and low-P supplies might result in some marked differentiations in the proportions of the total phosphorus which was present in the several organic forms. This presumption has been proven to be the case only to a very limited extent. The depression under low-P (table VII) in soluble organic-P, nucleic acid-P, and protein-P were on the whole very much alike; the average percentage reductions were respectively, 58, 50, and 56 %. Nucleic acid-P was reduced only slightly more by insufficient phosphorus than was soluble organic and protein phosphorus.

As the original high-P leaves grew older, there was a major reduction in inorganic phosphorus but little change occurred in the concentrations of any of the organic phosphate fractions, the only exception being in the reduction of nucleic acid-P from 0.24 to 0.16 %.

PHOSPHORUS FRACTIONS IN YOUNG OVULES AND MATURE SEED KERNELS: The concentrations of total, inorganic, soluble-organic, and nucleic acid-P in 10-day ovules (table VIII) with their attached and rapidly elongating lint hairs were all 40 %, or less, as high as those found in the leaves of young plants; protein phosphorus dropped to about one-tenth. On the other hand, lipid phosphorus which has been found to be less than 0.001 % in leaf tissues was present to the extent of 0.042 in the combined ovules and lint hairs.

In the mature seed kernels, soluble-organic phosphorus (soluble in 10 % trichloroacetic acid) was the prominent constituent; it constituted 75 % of total phosphorus. Inorganic and lipid phosphorus were about equally low and together constituted only 17 % of the total P; the final 8 % of the total was in the form of nucleic acid and protein P. The soluble-organic fraction included phytin which, as has been noted by others, is the major storage form of P in seed. The present data accord well with past results for cottonseed constituents as reviewed by Guthrie, Hoffpauer, Stansbury, and Reeves (9).

TABLE VII

PHOSPHORUS FRACTIONS ON COTTON LEAF BLADES FROM PLANTS GROWN WITHOUT BRANCHES IN WATER CULTURES

| COLLECTION | SAMPLE WT | | PHOSPHORUS AS % DRY WT | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | HIGH-P | LOW-P | INORGANIC | | SOLUBLE ORGANIC | | NUCLEIC ACID | | PROTEIN | | TOTAL ORGANIC | | TOTAL | |
| | | | HIGH | LOW | HIGH | LOW | HIGH | LOW | HIGH | LOW | HIGH | LOW | HIGH | LOW |
| *34 days** | | | | | | | | | | | | | | |
| All | 1.8 | 0.5 | 0.66 | 0.66 | 0.24 | 0.07 | 0.24 | 0.08 | 0.16 | 0.03 | 0.64 | 0.18 | 1.30 | 0.24 |
| *61 days*** | | | | | | | | | | | | | | |
| Old | 10.8 | 10.7 | 0.63 | 0.08 | 0.26 | 0.17 | 0.16 | 0.10 | 0.16 | 0.11 | 0.57 | 0.38 | 1.20 | 0.45 |
| New | 24.0 | 13.9 | 0.27 | 0.07 | 0.26 | 0.11 | 0.19 | 0.09 | 0.19 | 0.10 | 0.63 | 0.29 | 0.91 | 0.36 |
| *Wt means of all* | | | | | | | | | | | | | | |
| | ... | ... | 0.38 | 0.07 | 0.26 | 0.14 | 0.18 | 0.09 | 0.18 | 0.10 | 0.61 | 0.33 | 1.00 | 0.41 |

* Plants were kept for 34 days in high- and low-P.
** These high- and low-P plants were in high-P to day 34.

TABLE VIII

PHOSPHORUS FRACTIONS IN 10-DAY OVULES (WITH LINT HAIRS) AND IN MATURE SEED KERNELS. THE COMPOSITIONS OF THE LEAVES OF YOUNG PLANTS FROM TABLE VII ARE REPORTED FOR COMPARISON

| TISSUES | PHOSPHORUS FRACTIONS (% OF DRY WT) | | | | | |
|---|---|---|---|---|---|---|
| | INORGANIC | SOLUBLE ORGANIC | LIPID | NUCLEIC ACID | PROTEIN | TOTAL |
| Leaves of young plants | 0.66 | 0.24 | .... | 0.24 | 0.16 | 1.30 |
| Ovules (10-day) with lint hairs | 0.202 | 0.100 | 0.042 | 0.059 | 0.017 | 0.420 |
| Mature seed kernels | 0.080 | 0.700 | 0.083 | 0.047 | 0.025 | 0.935 |

## CONCLUSIONS AND SUMMARY

Upland cotton plants grown in sand and water cultures showed sharply depressed growth even though the phosphorus concentrations of a water culture was not allowed to fall below 2.7 ppm.

The reproductive growth of plants in sand cultures was inhibited by low phosphate to a greater extent than was vegetative development; the number and length of the fruiting branches were reduced and few bolls were set. Evidently the reproductive tissues did not have a high enough P-priority to give them an advantage over the vegetative tissue.

The phosphorus concentrations in various tissues of low-P sand-culture plants fell to a fifth or less of those in high-P plants (table II) even though enough phosphorus was being supplied to maintain some growth. In both high- and low-P solutions and in young as well as old plants, the leaves contained much more phosphorus than the stems and roots, but the concentrations in the latter fell off more rapidly with age on high-P than on low-P solutions.

On low-P solutions in sand cultures the concentration of reducing sugars and starch (table III) were in the order of four times greater on low-P than on high-P substrates, whereas sucrose (one exception) and hemicellulose showed only minor differences.

Estimates of the phosphorylation of the reducing sugars calculated on the basis of the phosphorus found in the alcoholic extracts for the sugar determinations indicated, as maximum values, an average of 50 % phosphorylation in the high-P plants but only 5 % in the low-P plants.

On low-P, the concentrations of nitrate and protein N (table IV) were both depressed whereas soluble-organic nitrogen was increased indicating a blocking of protein synthesis.

Under low-P nutrition there were rather consistent decreases in the percentages of calcium, magnesium, potassium, sulfur (table V). Iron percentages were not influenced. In accord with the views of others these depressions indicate a dependence, in mineral uptake, on phosphate-bond energy in the carrier molecules.

The roots of low-P plants (table I), like the stems, were low in phosphorus, but relative to top weight, the root growth was depressed less than top growth.

Phosphorus deficiency made its major symptomatic appearance in the form of large irregular blotches of leaf tissue (fig 1) which first whitened and then died.

Soluble-organic, nucleic acid, and protein phosphorus were measured (table VII) in the leaves of high- and low-P plants growing in water cultures. Under high-P these three fractions together constituted 62 % of the total phosphorus, whereas under low-P they constituted 83 % of the total. The concentration of total phosphorus under high-P was 1 % of the dry weight and under low-P 0.41 %. Relative to high-P, the soluble-organic P was reduced to 58 %, nucleic-acid P to 50 %, and protein P to 56 %. The three reductions are regarded as being notably similar indicating no major differentiations in the form of P (other than inorganic) which undergoes the greatest loss. At 61 days in this water-culture experiment, the P-deficiency of the substrate was only severe enough to reduce the weight of leaves by 25 %.

Comparisons (table VIII) of the phosphorus fractions in 10-day-old ovules, with attached lint hairs, with the leaves of 34-day-old plants on high-P water cultures (table VII) showed the ovules to have only one-third as much total phosphorus as the leaves; there were corresponding or larger reductions in the three organic fractions. Lipid phosphorus had been found to be less than 0.001 % in leaves but the ovules had 0.042 %. Mature seed kernels had over twice as high concentrations of total phosphorus and lipid phosphorus as the 10-day-old ovules.

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# CARBOHYDRATE DISTRIBUTION AS AFFECTED BY CALCIUM DEFICIENCY IN COTTON [1,2]

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Carbohydrate accumulation in various plant organs has often been associated with calcium deficiency. Groom (7) in 1896 suggested one function of calcium was to combine with oxalate ions to form calcium oxalate. Groom concluded that under conditions of calcium deficiency potassium oxalate retards the action of diastase and such plants tend to accumlatae starch. Nightingale et al (11) present analyses of whole tomato stems which they claim demonstrate the accumulation of carbohydrates by calcium deficient plants. They conclude this accumulation of carbohydrates resulted from the failure of calcium deficient plants to absorb and assimilate nitrate. Gauch (5) states that failure of bean plants to absorb nitrate ions when grown on solutions deficient in calcium was due to an unbalanced condition of the nutrient solution. Using Nightingale's solution, Gauch showed that bean plants deficient in calcium could not absorb nitrate ions; however, in a second series employing a more dilute nutrient solution, Gauch demonstrated that calcium deficient bean plants were able to absorb nitrate ions. Gauch concludes that the difference in nitrate absorption by plants in the two series is associated with the level of magnesium in the solutions thus showing that cationic imbalance was a major factor in Nightingale's observations.

Eckerson (4) showed that calcium among other factors was essential for the normal nitrate reductase activity of plants. Skok (12) theorized that if minus calcium plants lose their capacity to reduce nitrates, they are essentially minus nitrogen as well as minus calcium plants. Utilizing urea and nitrate as sources of nitrogen, Skok noted that minus calcium bean plants made much better growth when the reduced form of nitrogen was supplied. Hibbard and Grigsby (8) found that bean plants grown in solutions deficient in calcium continued to synthesize sugars and proteins but in lesser amounts than were produced by plants grown in complete nutrient solutions. Darkis et al (2) reported liming of soil resulted in a 21 % decrease in reducing and total sugars of tobacco. Thus existing reports on the role of calcium in the accumulation and distribution of carbohydrates in plants are in conflict. In light of those reports it seemed worthwhile to obtain more information concerning the influence of calcium deficiency on cotton.

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[2] Published with the approval of the Director of the Texas Agricultural Experiment Station as Technical Article No. 2466.

## Experimental Methods and Results

Plant Culture: Cotton seeds, strains Texas S9 and S3-2-3 were planted in flats containing washed creek sand. Twenty-five days after planting the seedlings were transplanted to 5-gallon jars containing nutrient solution. Three seedlings of each variety were placed in each of 18 jars. During the course of the experiment a modification of Hoagland's solution was employed. Each nutrient treatments received solutions containing the following salts.

| Salts | Conc, mM |
|---|---|
| $NH_4NO_3$ | 5 |
| $KNO_3$ | 5 |
| $MgSO_4 \cdot 7\,H_2O$ | 2 |
| $KH_2PO_4$ | 1 |
| NaCl | 10 |

In addition $CaCl_2$ was supplied to the three treatments as follows:

| Treatment | Conc $CaCl_2$, mM |
|---|---|
| I | 5.0 |
| II | 0.2 |
| III | 0.05 |

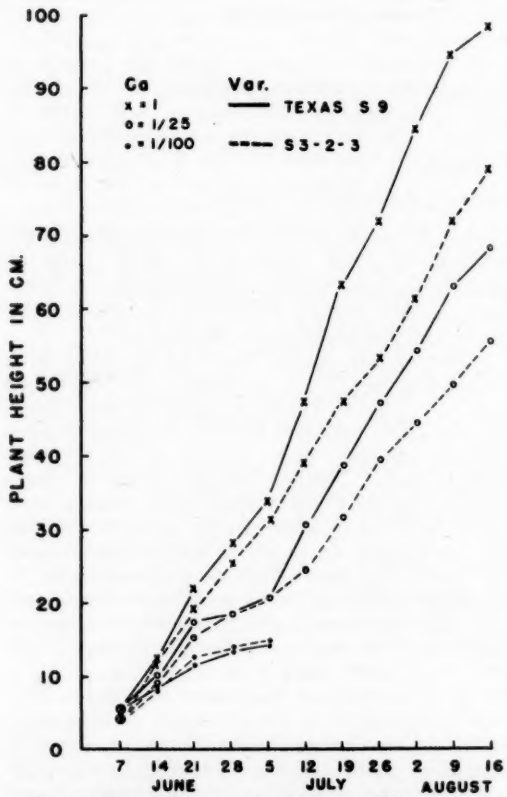



Fig. 1. The influence of substrate calcium on the growth of cotton as indicated by plant height.

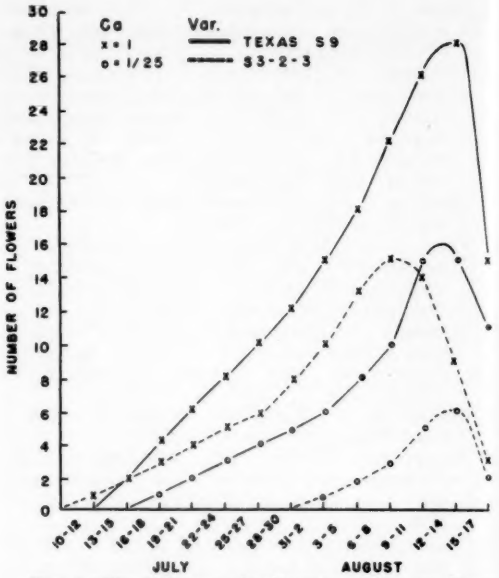



Fig. 2. The influence of substrate calcium on flowering of cotton. Figures given are the number of flowers produced/3 plants over a period of three days.

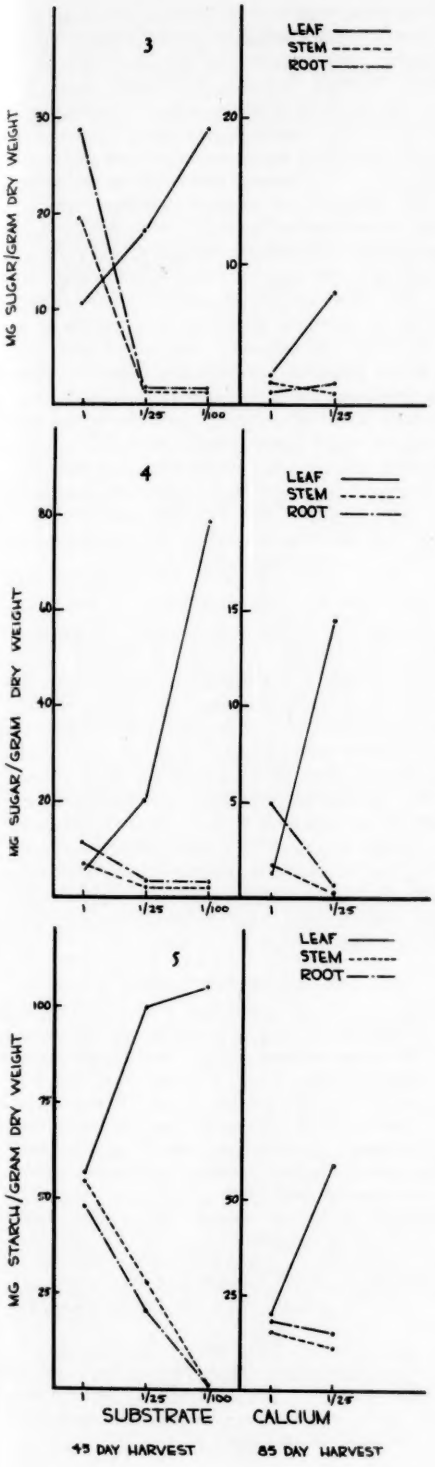
The above calcium levels were 1, 0.04, and 0.01 times that of Hoagland's solution. Because of the general availability of sodium to plants and since the present author (9) has shown that under conditions similar to those of this investigation sodium may partially substitute for calcium in the nutrition of cotton, it was felt advisable to add sodium chloride to the culture solutions. Minor elements were added to insure a sufficient supply. Iron was added as Versenol iron chelate at the rate of 2 ppm iron to all solutions. The pH of each solution was checked daily, and control was maintained between limits of 5.0 to 6.3 by the addition of dilute $HNO_3$ or $NH_4OH$.

Table I

Dry Weight of 45- and 85-Day Old Cotton Plants, Var. Texas S 9, As Influenced by Substrate Calcium

| Plant part | Dry wt, gm | | | | |
|---|---|---|---|---|---|
| | Ca level, 45-day harvest | | | Ca level, 85-day harvest | |
| | 1 | 0.04 | 0.01 | 1 | 0.04 |
| Leaf | 9.53 | 5.47* | 2.79** | 74.46 | 45.20* |
| Stem | 4.96 | 0.83* | 0.33** | 51.70 | 15.70* |
| Root | 4.68 | 1.38** | 0.51** | 21.46 | 10.76 |
| Fruit | .... | .... | .... | 121.53 | ....** |
| Total | 19.27 | 7.68* | 3.64** | 269.15 | 71.66** |

Figures given are average values for 6 plants.
* Difference between treatment and control (1) significant to 5 % level.
** Difference between treatment and control (1) significant to 1 % level.




All jars were drained, washed, and filled with freshly prepared solution each week. Each jar was supplied with a porous clay aerator attached to the central air line and a pressure of 5 lbs/in.$^2$ was maintained during the experiment. The experimental design was a randomized block with 3 replications on the greenhouse bench. Between 10:00 A.M. and 12:00 noon at 45 and 85 days after transplanting, plants from each treatment were harvested for fresh and dry weight determinations and carbohydrate analyses. Because of the limited growth it was necessary to harvest at 45 days all plants in the 0.01 treatment to insure sufficient material for chemical analysis. Height measurements and flower counts were made during the experiment.

Chemical Methods: Tissue which had been dried at 80° C to a constant weight in a forced draft oven was ground to pass an 80-mesh screen and, after extraction with 80 % alcohol, sugars and starch were determined by a modification of the Wildman and Hansen (14) method as employed by Eaton and Joham (3).

Plant Growth Response to Various Calcium Levels: Both strains, Texas S9 and S3-2-3, responded in like manner to variations in substrate calcium. Height, number of flowers, and dry weight were directly related to substrate calcium (figs 1, 2 and table I). The number of days from planting to appearance of the first flower was inversely related to substrate calcium. This relation is particularly noticeable in strain S3-2-3 (fig. 2). Substrate calcium had little or no influence on the date of maximum flower production; however, a marked influence of calcium on boll set was noted since at the second harvest no fruit were set on the 0.04 plants (table I).

Influence of Substrate Calcium on Carbohydrate Distribution: In calcium deficient plants carbohydrates accumulated in the leaves while extremely low levels were noted in the stems and roots, and in some instances were not detectable by the analytical methods employed. Again, as in growth, both strains responded in a like manner thus only the data for Texas S9 are presented in figures 3, 4, and 5. Reducing sugars, sucrose, and starch of the leaves were inversely related to substrate calcium at both harvest dates while a direct relation was observed in the stems and roots (figs 3, 4, 5).

At 45 days reducing sugars and sucrose in the organs of the high calcium treated plants increased in the following order: leaves, stems, roots. The inverse of the above order tended to be the case in the 0.04 and 0.01 calcium treated plants with the leaves always containing the highest concentrations of reducing sugars, sucrose and starch. Little trend was noted for starch concentration in the plant parts of the high calcium treated plants.

Figs. 3 to 5. The concentration of carbohydrates in various parts of the cotton plant as influenced by substrate calcium. Fig. 3 (*top*). Reducing sugars. Fig. 4 (*middle*). Sucrose. Fig. 5 (*bottom*). Starch.

From 45 to 85 days a marked reduction in carbohydrates of the leaves occurred in all treatments, although the relation between calcium deficiency and carbohydrate accumulation in the leaves was still apparent. During the period from 45 to 85 days carbohydrates in the stems and roots of the high calcium treated plants decreased while in the same period an increase was noted for reducing sugars and starch in the same organs of the 0.04 plants. During that period the high calcium plants set a large boll crop while no fruit were set by the 0.04 plants.

## Discussion

Carbohydrate accumulation is often associated with limitation of growth by nutrient deficiencies provided photosynthesis has not been impaired. Growth limiting levels of calcium supplied to cotton resulted in increased carbohydrate concentrations in the leaves and decreased levels in the stems and roots. Thus, calcium deficiency differs widely from the effect of nitrogen deficiency on carbohydrate distribution in cotton. In the latter case, according to Wadleigh (13), carbohydrates accumulate in all tissues of the cotton plant. The initial influence of nitrogen deficiency is a limitation of growth resulting from a reduction in protein synthesis. Snythesis and translocation of carboyhdrates are not greatly influenced by nitrogen deficient levels above those producing chlorosis. Calcium deficiency, on the other hand, is expressed in a different manner. From data presented here it seems likely that one factor associated with the limitation of growth by calcium deficiency is the failure of normal carbohydrate transport. The marked accumulation of carbohydrates in the leaves indicates synthesis of these materials continues under calcium deficient conditions which severely limit growth.

On first analysis of the paper by Nightingale et al (11) and the data presented here, it would seem that tomato and cotton respond differently to calcium deficiency as far as the distribution of carbohydrates is concerned. They (11) stated that carbohydrates accumulated in the stems of calcium-deficient tomato plants. Examination of their data makes their conclusion questionable. In their experiment, they employed tomato plants which had grown for several weeks in four-inch pots, and at the time of treatment application the plants were already high in carbohydrates. During the course of the experiment little or no change occurred in the stem carbohydrates of the minus calcium plants as shown by their initial and final analyses. Considering that very little growth took place it seems likely that their data do not represent an accumulation of carbohydrates in the stems of the minus-calcium plants but rather a failure of carbohydrate translocation and utilization under calcium deficiency. Thus, the present data on cotton and those presented by Nightingale et al on tomato may actually be in close agreement.

The limitation of amylase activity with calcium deficiency as suggested by Groom (7) does not seem to be a factor in the failure of carbohydrate transport or starch accumulation by calcium deficient cotton plants. Marked increases in reducing sugars and sucrose in leaves of calcium deficient plants and deficiencies of these materials in stems and roots indicates factors other than amylase activity are responsible for inhibition of the normal translocation mechanism.

The influence of calcium deficiency on carbohydrate distribution in cotton is very similar to the effect of boron deficiency in tomato plants (10). Gauch and Dugger (6) suggested that calcium also could affect the translocation of carbohydrates through its relation to boron as reported by Brennan et al (1). In the latter publication it was noted that increased substrate calcium accentuated boron deficiency. Thus according to the reasoning of Gauch and Duggar, high levels of substrate calcium in the presence of low levels of boron would cause boron deficiency and thus influence the carbohydrate distribution. In the present paper calcium deficiency was noted to limit carbohydrate translocation, therefore, it seems likely that the role of calcium in transport of carbohydrates may be independent of boron.

## Summary

Growth and fruiting of cotton as measured by height, dry weight, number of flowers produced, weight of bolls and earliness to flower were directly related to substrate calcium. An inverse relation was noted between leaf carbohydrates and calcium availability while carbohydrate levels of stems and roots tended to be directly dependent on substrate calcium. Such a distribution pattern was considered to be a result of the failure in carbohydrate translocation due to calcium deficiency. A comparison was made between the results of calcium and nitrogen deficiency on cotton. Both calcium and boron deficiencies were noted to exert similar influences on the distribution of carbohydrates.

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# EFFECT OF 6-(SUBSTITUTED)THIO- AND AMINO-PURINES ON GERMINATION OF LETTUCE SEED[1]

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The biological activity of 6-(2-furfuryl)aminopurine (kinetin), a substance which has been found to effect increased cell division in tobacco "callus" tissue (8), has led to the synthesis and biological study of a number of 6-(substituted)purine derivatives. Several of these compounds have recently been found to have pronounced biological activity in both plant and animal systems. For example, in the moss *Tortella caespitosa*, 6-(2-thenyl)aminopurine, 6-benzylaminopurine and 6-*n*-pentylaminopurine have been found to have effects equal to or slightly greater than that of kinetin on the development of gametophores (5). Further, some of the purine analogues such as 6-($\omega$-phenylheptyl)aminopurine were found to be several thousand fold more active than adenine and several hundred fold more active than kinetin in retarding tentacle regeneration in hydra (6, 10).

The use of various organic compounds (especially auxins) in an attempt to increase the rate of germination of seeds has been studied for many years (1); however, only limited success has been obtained (3). In view of the biological activity of these purine derivatives, a study was made of their effects on seed germination.

The effect of several compounds, especially thiourea, upon lettuce seed germination has been reported by Thompson and Kosar (13), and during the final stages of preparation of this manuscript an article appeared (7) which presented some data on stimulation of lettuce seed germination by 6-(2-furfuryl)-aminopurine as well as some other 6-(substituted)-aminopurines. These latter results were interrelated to the red light effect previously observed as a requirement for lettuce seed germination (2). We have also observed such an effect with 6-(substituted)thiopurines (12).

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## MATERIALS AND METHODS

Several types of field variety seed were initially studied in an effort to determine the effect of pre-soaking in 6-(substituted)purine solutions on their germination. In several instances enhanced rates of germination were noted; however, the most readily adaptable seed found for this study was a variety of lettuce (Early Curled Simpson).

Most of the compounds studied are relatively new, and their method of synthesis are reported elsewhere (4, 6, 9 to 12).

Unless otherwise noted, 100 or more seeds were pre-soaked in 100 ml of each purine solution, made up in distilled water at a concentration of 10 $\mu$gm/ml, for a period of 8 hours. In order to minimize light effects, the soaking period was uniformly overnight, and a water control was always included under identical experimental conditions as a standard. The seeds were drained of excess solution on filter paper, and finally placed on filter paper, wet with the corresponding purine solution, in Petri dishes and allowed to germinate in a dark room at 30° C. The same relative results were obtained whether the initial manipulation of seeds was carried out in diffuse light or in blue light (fluorescent light filtered through four layers of du Pont 300 MSC dark blue cellophane). The temperature of 30° C was chosen because these lettuce seed, soaked in water alone, germinate very slowly at this temperature in the dark. In order to show that the inactive compounds were not inhibiting germination, a corresponding experiment was performed so that germination was allowed to proceed in the presence of continuous fluorescent light at 25° C.

The light and temperature requirements for this variety of lettuce seed, with respect to rate of germination using several of the more "active" compounds, was studied. The seeds were pre-soaked in water, or

TABLE I

EFFECT OF VARIOUS 6-(SUBSTITUTED)PURINES ON THE RATE OF GERMINATION OF LETTUCE SEED

| COMPOUND ** | % SEED GERMINATED AT GIVEN NO. OF HRS* | | | | NO. HRS REQUIRED FOR 30% GERMINATION IN THE DARK |
|---|---|---|---|---|---|
| | 24 | 36 | 48 | 60 | |
| *6-(n-Alkyl)thiopurines* | | | | | |
| (Water blank control) | 0 (39) | 0 | 9 (71) | 11 | >84 |
| Butyl- | 6 (34) | 11 | 17 (72) | 22 | 84 |
| 3-Methylbutyl- | 1 (62) | 7 | 11 (94) | 18 | >84 |
| Pentyl- | 31 (59) | 55 | 73 (81) | 77 | 24 |
| Hexyl- | 29 (55) | 59 | 75 (85) | 79 | 24 |
| Heptyl- | 5 (44) | 10 | 14 (79) | 18 | >84 |
| *6-(n-Alkyl)aminopurines* | | | | | |
| (Water blank control | 0 (38) | 2 (70) | 2 (71) | 5 (78) | >84 |
| Propyl- | 39 (67) | 62 (83) | 69 (89) | 72 (92) | 20 |
| Butyl- | 65 (58) | 83 (78) | 86 (82) | 90 (82) | 11 |
| Pentyl- | 55 (60) | 72 (78) | 85 (79) | 89 (83) | 12 |
| Hexyl- | 59 (57) | 76 (79) | 79 (81) | 83 (86) | 12 |
| Heptyl- | 50 (52) | 67 (70) | 77 (74) | 82 (80) | 14 |
| *6-(Di-alkyl)aminopurines* | | | | | |
| (Water blank control) | 1 (17) | 1 (58) | 2 (83) | 3 (88) | >84 |
| Di-propyl- | 11 (42) | 27 (66) | 41 (80) | 47 (82) | 38 |
| Di-butyl- | 7 (38) | 17 (65) | 25 (84) | 30 (89) | 60 |
| Di-pentyl- | 9 (31) | 10 (53) | 19 (75) | 22 (81) | >84 |
| *6-(Phenylalkyl)thiopurines* | | | | | |
| (Water blank control) | 0 (49) | 3 (79) | 3 | 6 | >60 |
| Phenylmethyl- | 3 (63) | 55 (82) | 63 | 72 | 30 |
| 2-Phenylethyl- | 9 (50) | 33 (72) | 41 | 48 | 34 |
| 3-Phenylpropyl- | 5 (50) | 45 (73) | 55 | 62 | 31 |
| 5-Phenylpentyl- | 1 (50) | 2 (79) | 3 | 3 | >60 |

TABLE I (*Continued*)

| COMPOUND ** | % SEED GERMINATED AT GIVEN NO. OF HRS* | | | | NO. HRS REQUIRED FOR 30% GERMINATION IN THE DARK |
|---|---|---|---|---|---|
| | 24 | 36 | 48 | 60 | |
| *6-(Phenylalkyl)aminopurines* | | | | | |
| (Water blank control) | 0 (49) | 3 (79) | 3 | 6 | >60 |
| Phenylmethyl- | 35 (62) | 86 (75) | 87 | 87 | 22 |
| 2-Phenylethyl- | 35 (52) | 70 (76) | 77 | 81 | 23 |
| 3-Phenylpropyl- | 23 (53) | 71 (72) | 77 | 83 | 26 |
| 4-Phenylbutyl- | 1 (30) | 5 (66) | 5 | 10 | >60 |
| *6-(Substituted)aminopurines* | | | | | |
| (Water blank control) | 0 (29) | 0 | 0 (50) | 0 | >84 |
| α-Naphthyl- | 17 (67) | 71 | 87 (83) | 89 | 26 |
| α-Naphthyl-methyl- | 25 (72) | 78 | 86 (85) | 88 | 25 |
| 2-α-Naphthyl-ethyl- | 0 (35) | 0 | 1 (52) | 1 | >84 |
| 5-α-Naphthyl-pentyl- | 0 (36) | 2 | 3 (59) | 5 | >84 |
| 2-Furfuryl- | 30 (58) | 56 | 81 (78) | 82 | 24 |
| 2-Thenyl- | 17 (71) | 56 | 85 (83) | 88 | 28 |
| 2-Pyridyl-methyl- | 24 (52) | 67 | 88 (74) | 89 | 25 |
| 3-Pyridyl-methyl- | 17 (66) | 69 | 89 (83) | 94 | 27 |
| 4-Pyridyl-methyl- | 4 (53) | 56 | 78 (75) | 79 | 30 |

* The values given in parentheses were obtained using continuous fluorescent light during germination; the other values were obtained after germination in the dark.

** The following 6-(substituted)thiopurines were tested and found to be relatively inactive in stimulating seed germination under the test conditions: methyl-, ethyl-, propyl-, 2-methylpropyl-, octyl-, decyl-, cetyl-thiopurine; the following 6-(substituted)aminopurines were also relatively inactive: methyl-, ethyl-, decyl-, dimethyl-, diethyl-, 5-phenylpentyl-, 7-phenylheptyl-, and 11-phenylundecyl-aminopurine.

the purine solution, in total darkness for eight hours; after which, they were transferred to Petri dishes in the presence of blue light, and the rate of germination at 22° and 30° C in total darkness and in the presence of continuous fluorescent light determined.

The number of seeds germinated at various time intervals was determined by visual observation of the appearance of the radicle, and all of the "dark germination" readings were made in the presence of blue light. The germinated seeds were removed from the Petri dishes after each time interval to facilitate counting.

## RESULTS

From the germination data presented in table I it is apparent not only that certain 6-(substituted)-aminopurines possess a "dormancy breaking" activity, but that these effects are also produced by 6-(substituted)thiopurines as well. Further, at a concentration of 10 μgm/ml, the activity within the thio- and amino- series both reach peak activity near the same carbon length of the side chain. The activity of the compounds in the alkylthio- series peaks much more sharply with increase in chain length than in the alkylamino- series; however, the most active com-

TABLE II

EFFECT OF VARIOUS CONCENTRATIONS OF 6-(ALKYL)THIOPURINES ON RATE OF GERMINATION OF LETTUCE SEED IN THE DARK

| COMPOUND, 6-(ALKYL)THIOPURINE | CONC. μGM/ML | % SEED GERMINATED AT GIVEN NO. OF HRS | | | | |
|---|---|---|---|---|---|---|
| | | 22 | 46 | 69 | 90 | 141 |
| (Water blank control) | .. | 0 | 6 | 9 | 10 | 12 |
| Butyl- | 1 | 2 | 7 | 9 | 11 | 12 |
| | 3 | 0 | 6 | 7 | 9 | 10 |
| | 10 | 5 | 16 | 19 | 21 | 22 |
| | 30 | 4 | 55 | 67 | 71 | 75 |
| Pentyl- | 1 | 2 | 5 | 8 | 11 | 13 |
| | 3 | 3 | 25 | 29 | 31 | 32 |
| | 10 | 18 | 65 | 71 | 77 | 77 |
| | 30 | 3 | 71 | 84 | 86 | 87 |
| Hexyl- | 1 | 2 | 6 | 7 | 9 | 12 |
| | 3 | 3 | 13 | 15 | 18 | 20 |
| | 10 | 12 | 57 | 69 | 76 | 80 |
| | 30 | 16 | 74 | 83 | 90 | 91 |
| Heptyl- | 1 | 0 | 0 | 2 | 4 | 8 |
| | 3 | 0 | 3 | 7 | 9 | 10 |
| | 10 | 1 | 5 | 9 | 11 | 11 |
| | 30 | 3 | 10 | 15 | 20 | 20 |

pounds in both series are centered at the pentyl- and hexyl- carbon chain length.

The 6-(phenylalkyl)thio- and 6-(phenylalkyl)-aminopurines are comparable in their effect on altering the rate of seed germination under the experimental conditions studied. The most active compounds in both instances were the phenylmethyl-, phenylethyl- and phenylpropyl- compounds. When the phenyl- group was replaced by an α-naphthyl- radicle only the 6-(α-naphthylmethyl)aminopurine was appreciably active in stimulating the rate of lettuce seed germination at a concentration of 10 μgm/ml.

Substitution of a heterocyclic group for the aromatic nucleus in these 6-(substituted)aminopurines resulted in an increased germination rate of the same order of magnitude as obtained in the alkyl- and phenylalkyl- series above. The active compounds include 6-(2-furfuryl)aminopurine, 6-(2-thenyl)aminopurine and 6-(α-, β- and γ-pyridylmethyl)aminopurines.

The effect of concentration of the purine solutions on the rate of germination was not studied with every compound. A concentration range of three-fold increments was tested in the alkylthio- series, and the results indicated that a concentration of 10 μgm/ml of purine solution was adequate to initiate activity. At higher concentrations, as noted in table II, the analogues with shorter or longer chain length than the more active compounds possessed some effect on the rate of germination.

The light and temperature requirement for this variety of lettuce seed, with respect to rate of germination after pre-soaking in water and in the purine solutions, has been demonstrated with several of the more "active" compounds. In the dark at 22° C, seeds pre-soaked in a 10 μgm/ml solution of 6-benzylaminopurine, for example, were found to be 71 % germinated at 60 hours compared to 76 % germination for seeds soaked in water alone. However, at 30° C the corresponding values were 59 and 9 %, respectively. A similar increased rate in germination at the higher temperature in the absence of light was noted with several of the other compounds (table III). Further, some of these compounds appear to increase the rate of germination of these seeds even in the presence of light. For example, after 48 hours

TABLE III

STUDY OF LIGHT-TEMPERATURE EFFECTS OF CERTAIN 6-(SUBSTITUTED)PURINES ON GERMINATION OF LETTUCE SEED

| COMPOUND, 6-(SUBSTITUTED)PURINE | TEMP, °C | % SEED GERMINATED AT GIVEN NO. OF HRS | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 24 | | 36 | | 48 | | 60 | |
| | | LT. | DK. | LT. | DK. | LT. | DK. | LT. | DK. |
| (Water blank control) | 22 | 1 | 4 | 18 | 17 | 45 | 57 | 67 | 76 |
| | 30 | 0 | 0 | 3 | 1 | 8 | 5 | 30 | 9 |
| Pentylthio- | 22 | 4 | 5 | 13 | 28 | 45 | 56 | 54 | 65 |
| | 30 | 7 | 1 | 27 | 8 | 48 | 28 | 58 | 59 |
| Butylamino- | 22 | 14 | 10 | 29 | 24 | 54 | 53 | 66 | 59 |
| | 30 | 25 | 2 | 44 | 13 | 60 | 46 | 64 | 59 |
| Benzylthio- | 22 | 3 | 8 | 8 | 19 | 33 | 51 | 46 | 59 |
| | 30 | 9 | 4 | 33 | 11 | 55 | 44 | 63 | 60 |
| Benzylamino- | 22 | 15 | 21 | 25 | 40 | 43 | 61 | 48 | 71 |
| | 30 | 8 | 18 | 29 | 36 | 43 | 48 | 51 | 59 |
| α-Naphthylmethylamino- | 22 | 11 | 11 | 30 | 34 | 44 | 55 | 51 | 61 |
| | 30 | 17 | 0 | 27 | 9 | 46 | 38 | 56 | 52 |
| 2-Furfurylamino- | 22 | 15 | 9 | 27 | 21 | 41 | 43 | 54 | 58 |
| | 30 | 7 | 10 | 29 | 30 | 45 | 49 | 60 | 57 |

lettuce seed pre-soaked in water and kept at 22° C were 45 % germinated compared to only 8 % germination for seeds kept at 30° C; which compares with 43 % germination at both temperatures for seed pre-soaked in 6-benzylaminopurine (table III).

The unsubstituted parent compounds, 6-mercaptopurine and adenine, had no appreciable effect on the germination of the lettuce seed over that of those seeds soaked in water alone.

## Discussion

Many of the 6-(substituted)purines studied appreciably increased the rate of germination of lettuce seeds under the conditions of the experiments. That the "inactive" compounds were not toxic was indicated by the control experiment in the presence of light which allowed germination after pre-soaking in these compounds at approximately the same rate as the water controls.

The "active" compounds appear to have the property of replacing, or, in some manner, overcoming the light requirement in stimulating lettuce seed germination. The stimulation of germination by these compounds is most readily observed in the dark at 30° C. In the presence of light or at a lower temperature (22° C) lettuce seed germinate so rapidly that only moderate stimulatory effects of the purine derivatives are observed under these conditions.

These compounds probably exert some definite control relative to the biological effects of light and change of temperature. An increased rate of germination at 30° C was observed by increasing the concentration level of the more active purine analogues, resulting in very good dose-response curves of the data (table II). Also, in the presence of light, the rate of germination observed with lettuce seeds soaked in several of the more "active" purine solutions was increased compared with that of the water controls at 30° C, as indicated in tables I and III.

The fact that both the 6-(substituted)thio- and aminopurines affect the rate of germination of lettuce seed indicates that structural specificity necessary for this biological activity is not very exacting. It was observed that the number of "active" 6-(substituted)-purine analogues, within a homologous series, was greater in the amino- than in the thio- series suggesting that the 6-(substituted)aminopurines are possibly more closely related to the natural substance(s), which are involved in the normal seed germination, than are the thio-homologues. The maximum activity in both series was obtained with alkyl chain lengths of 5 to 6 carbon atoms or with an aromatic or heterocyclic substituent connected by a one or two carbon side chain.

## Summary

Several 6-substituted amino- and thiopurines have been found to stimulate the rate of lettuce seed (Early Curled Simpson) germination. The size of the substituent group on the aminopurines is less specific for this activity than is the size of the group on the thiopurine; however, the maximum activity in both series is obtained with alkyl chain lengths of 5 to 6 carbons or with an aromatic or heterocyclic substituent connected by a methyl or ethyl group. The stimulation of lettuce seed germination by these compounds is most readily observed in the dark at 30° C. In the presence of light or at a lower temperature (22° C), lettuce seed germinate so rapidly that only moderate effects of the purines derivatives are observed under these conditions.

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# EFFECTS OF ATMOSPHERIC FLUORIDE ON THE RESPIRATION RATE OF BUSH BEAN AND GLADIOLUS LEAVES[1]

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Many atmospheric pollutants that occur as a result of industrialization are detrimental to plant life. Gaseous hydrogen fluoride is one of the most common of these, and is probably the most phytotoxic of the halogen compounds occurring as atmospheric impurities. Plants that have been exposed to excess fluorides exhibit a characteristic "scorch" of the leaf tip or margin. The affected tissue initially appears as a gray water-soaked area at the leaf apex, margin, or more rarely in intercostal regions. This tissue may then rapidly desiccate to leave a dried brown to buff colored area called scorch (7). In some instances chlorosis may occur prior to the "water-soaked" stage (16).

Numerous studies have been made on plants grown in the field in the presence of atmospheric fluorides (2, 4, 7, 9, 13, 16), and some controlled fumigation experiments have been conducted (3, 6, 12, 17). In most of these investigations, the degree of visible leaf damage has been correlated with the amount of atmospheric fluorides and the concentration of accumulated tissue fluorides. In this manner the fluoride tolerance of many species and varieties has been established.

Although it is well known that fluorides inhibit phosphatase, enolase and amylase enzyme systems (15), few studies have been made on the physiological affects of sub-lethal concentrations of fluorides on leaf tissue. Katz and Shore (18) stated that atmospheric fluorides inhibit photosynthesis and inactivate phosphatase, enolase and amylase systems, but offered no data to support this contention. They also inferred that acid gases convert chlorophyl molecules into phaeophytins, and presented data for the effects of sulfur dioxide but not for fluorides.

Because of the sparsity of information on the physiological effects of sub-lethal concentrations of fluorides on plants, it was felt that such an investigation should be undertaken. This paper reports the results of some preliminary studies of the effects of atmospheric fluorides on the respiration of leaf tissue.

## MATERIALS AND METHODS

Bush beans (var. Burpee Tender Pod) and gladioli (var. Snow Princess) were used in these experiments. A fumigation chamber was constructed of a Tygon coated wood frame covered with polyvinylite plastic; which was sealed to the frame with a polyvinyl cement making the chamber air tight. Air was bubbled at a constant volume rate through a bottle of distilled water, then through a polyethylene bottle containing 0.274 *N* HF, and finally through a heated polyethylene tube leading to the forced air stream entering the chamber. The bottles and their contents were maintained at 40° C by keeping them immersed in a constant temperature water bath. The air stream entering the chamber changed the air every forty-five seconds. This volume was sufficient to maintain the temperature and humidity inside of the chamber the same as the outside air. The air stream was baffled so that no movement of the leaves was apparent.

The polyvinylite plastic reduced the light intensity 18 % at 400 mμ and 12 % at all wavelengths from 450 to 700 mμ.

Air from inside of the chamber was sampled by bubbling through a column of distilled water for seven out of every thirty minutes of fumigation. The water was changed every three days and analyzed for fluorides by a modification of the method described by Nielsen and Dangerfield (14). The modification consisted of titrating the concentrated eluterate to a specific end point as determined with a spectrophotometer.

The bean seeds were germinated in vermiculite. Soon after germination, seedlings were selected for uniformity of size of the first true leaf and transplanted into vermiculite in separate plastic containers. They were watered daily with 80 % Hoagland's solution. The gladioli were grown from number one corms placed in vermiculite and watered with 80 % Hoagland's solution.

When the bean plants were 25 days old, half of them were fumigated continuously at 30 ppb fluoride and half were placed outside of the chamber and used as control plants. Leaf samples were taken after intervals of fumigation and respiration measured manometrically. Six leaves from control and fumigated plants were collected at the time of measurement and two discs, 1.6 cm in diameter, were punched from each leaf midway between the base and apex and between the midrib and leaf margin. Four leaf discs were placed on moist filter paper in each of three Warburg reaction vessels. The discs were placed on one edge and leaned against the center well. After an equilibrium period of 15 minutes, the respiration rate was measured. The bath temperature was 28.5° C.

One half of the gladiolus plants were sprayed thoroughly with a 16.8 % solution of calcium hydroxide containing 0.12 % casein and 0.06 % Triton B-1956. The purpose of this spray was to precipitate the atmospheric fluorides on the leaf surface, thus preventing their entrance into the leaf. The sprayed plants thus acted as a control for the unsprayed plants when both were exposed to atmospheric fluorides. In order to determine whether the spray had any effect on leaf respiration, half of the sprayed plants were placed outside of the chamber with unsprayed control plants. It had been demonstrated earlier that the respiration rate of the leaf tissue 10 to 20 cm from the apex of

[1] Received September 20, 1956.

TABLE I

RESPIRATION RATE MEASUREMENTS OF FUMIGATED AND CONTROL TISSUE (BUSH BEAN)

| TREATMENT | DAYS OF FUMIGATING | MEAN OF $\mu$L $O_2$ CONSUMED/HR PER 100 MG DRY WT WITH STANDARD ERROR | DIFFERENCE IN RESPIRATION RATES OF CONTROL AND FUMIGATED TISSUE, % |
|---|---|---|---|
| Fumigated | 8 | 292.00 ± 4.33 | 58.44 |
| Control | 8 | 184.35 ± 9.75 | |
| Fumigated | 12 | 471.00 ± 7.64 | 74.44 |
| Control | 12 | 270.00 ± 19.60 | |
| Fumigated | 18 | 489.30 ± 8.80 | 7.47 |
| Control | 18 | 455.30 ± 12.50 | |

the leaf was highly constant. Consequently, the plants were fumigated until the leaves were scorched down into this area. At this time respiration rate measurements were made on tissue strips immediately adjacent to the scorched tissue and also 5 cm down from the dead area in apparently healthy tissue.

Three experiments were conducted: 1) Bush bean plants were fumigated for 18 days at 30 ppb atmospheric fluoride concentration. Respiration rate measurements were made after 8, 12 and 18 days of fumigation. 2) Bush bean plants were fumigated for 14 days at 30 ppb atmospheric fluoride concentration. Respiration rate measurements were made on the young, middle age and old leaves. 3) Gladiolus plants were fumigated for 25 days at 2.4 ppb atmospheric fluoride concentration. Respiration rate measurements were made on tissue adjacent to and 5 cm basipetally from the scorched front.

## RESULTS

1. Respiration of bush bean leaves after 8, 12 and 18 days fumigation (30 ppb). Table I shows the results of the leaf respiration measurements. After eight days of fumigation the respiration rate of the fumigated plants was 58 % higher than that of the non-fumigated, control plants. After twelve days the leaves exhibited slight intercostal chlorosis and the difference in respiration increased to 74 %. The chlorosis was more severe after eighteen days of fumigation but the respiration rate was still 7 % higher than that of the healthy green leaves of the control plants. At this time the apical buds were dead and stem elongation had ceased.

2. The effect of fluoride fumigation on young, middle age and old leaves. At the beginning of the fumigation period, the leaves at the sixth node had just started to expand while those at the third node had apparently just reached full size. These were used as the young and middle age leaves respectively. The old leaves were taken from the second node. Respiration rate measurements were made on these leaves

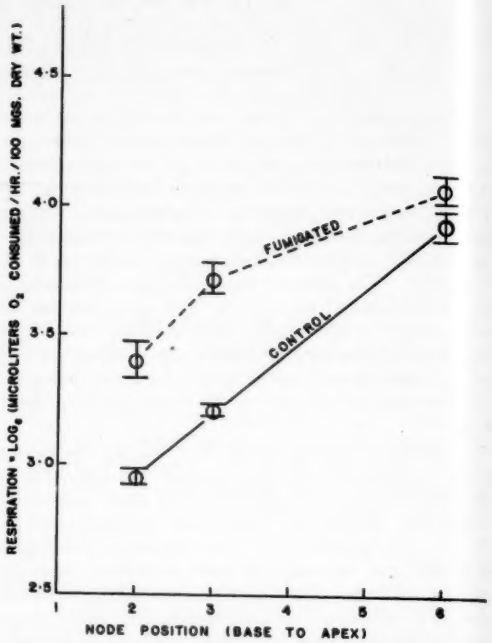


FIG. 1. A comparison of the respiration rates of fumigated and control leaves taken from the 2nd, 3rd and 6th nodes. The distance between the horizontal bars at each point indicates the standard error.

at the end of 14 days of fumigation, before any visible leaf scorch had appeared, although intercostal chlorosis was evident. The respiration rate of leaves of the control plants increased with node position toward the apex. The rate of respiration of the fumigated leaves was 58 % higher in the older leaves, 66 % higher in the middle age leaves and almost 8 % higher in the young leaves (fig 1).

3. After fumigation the unlimed leaves of gladiolus showed the characteristic tip scorch with a chlorotic region of about 3 to 5 mm between the brown and

TABLE II

RESPIRATION RATES OF FUMIGATED LIMED AND UNLIMED AND OF CONTROL LIMED AND UNLIMED SNOW PRINCESS GLADIOLUS LEAVES

| TREATMENT | MEAN OF $\mu$L $O_2$ CONSUMED/HR PER 100 MG DRY WT WITH STANDARD ERROR | INCREASE IN RESPIRATION RATE DUE TO FLUORIDES, % |
|---|---|---|
| Control—unlimed ..... | 99.11 ± 3.18 | |
| Control—limed ..... | 100.90 ± 5.06 | |
| Fumigated-limed ..... | 108.15 ± 4.05 | 6.65 |
| Fumigated—unlimed chlorotic tissue next to burned front ..... | 160.00 ± 1.47 | 61.40 |
| Fumigated—unlimed 5 cm from burned front .... | 118.47 ± 6.73 | 19.60 |

the green, apparently healthy tissue. In some cases, the scorch followed the intercostal areas leaving veinous regions green. However, the general rule was that the burning cut across veinous areas almost as readily as across intercostal areas. Table II shows the results of the respiration rate measurements.

Coating the leaves with a lime layer had no affect on the respiration rate of plants growing outside of the chamber. The respiration rate of the highly chlorotic tissue next to the scorched areas on fumigated plants was 61.4 % higher than that of tissue from unlimed control plants. Green tissue 5 cm from the scorched area showed a 19.6 % higher respiration rate than unlimed control plants. Fumigation increased the respiration rate of limed leaves 6.7 %.

## Discussion

It is obvious from these data that the presence of sub-lethal concentrations of fluorides in the leaf tissue of bush bean and gladiolus caused an increase in respiration when measured as a function of oxygen uptake.

The magnitude of this increase in respiration of bush bean leaves fumigated with fluorides became greater with time of exposure up to a certain point and then decreased. This was correlated with visible damage of the tissue. After 8 days of fumigation clear watery spots appeared and the respiration rate was 58 % above that of the controls. At 14 days the tissue was beginning to show slight chlorosis and the rate was 74 % higher. Severe chlorosis appeared at 18 days and the increase in respiration was only 7 %. Assuming that the tissue fluoride concentration became higher with time of exposure (9, 10), the respiration rate increased with fluoride concentration up to a certain level and rapidly declined approaching death of the tissue.

In gladiolus, the chlorotic tissue next to the scorched area showed a much higher respiration rate than apparently healthy tissue several millimeters away from the visibly damaged area. Thomas (15) states that fluorides are translocated to the tips of gladiolus leaves, and there is a steep concentration gradient of fluorides from the burned margin down the leaf. Thus, it appears that the degree of increased respiration in gladiolus was also correlated with tissue fluoride concentration.

Fluorides are usually considered to be respiratory inhibitors (5), and the reason for the apparent increase in respiration noted in these experiments is not understood. Field studies made by these authors (11) of the respiration of peach and pear leaves from plants growing in a fluoride atmosphere, indicated a converse situation, i.e., the leaf respiration was inhibited when compared to lime sprayed controls. However, the atmospheric fluoride level was too low to produce any visible symptoms in these species during the experiment. If the data from these two separate studies on different species are considered together, the inference might be made the fluorides might cause an initial inhibition of respiration when at low concentrations, followed by a stimulation of respiration with the appearance of visible damage due to higher concentrations. Experiments are now in progress to clarify this generalization.

Fluoride fumigation had more effect on stimulating the respiration of middle age and older leaves of bush bean than of the younger leaves. Daines (3) reported that middle age leaves showed visible damage earlier than young leaves. Thus, the middle age and older leaves may have shown a greater increase in respiration rate because of higher tissue fluoride concentrations.

Allmendinger et al (1), demonstrated the protective effect of lime spray on gladiolus. The fumigated plants that had been sprayed with lime showed only a slight increase in respiration rate over nonfumigated sprayed plants. This indicates that the lime spray is quite effective in precipitating atmospheric fluorides on the leaf surface. The lime spray itself did not affect the respiration rate of the gladiolus, however, peaches, pears and prunes showed a definite inhibition of respiration when sprayed with lime (11).

## Summary

Bush beans and gladioli were exposed to known concentrations of atmospheric fluoride and the respiration rates of their leaves was measured manometrically. The fumigation resulted in an increase in leaf respiration of bush bean plants after a minimum of 8 days at 30 ppb atmospheric fluoride concentration. Middle age and old leaves showed a greater increase in respiration than did young leaves. Gladiolus leaves fumigated at 2.4 ppb for 25 days showed a high degree of stimulation of respiration of the chlorotic tissue next to the scorched area, and a lower but significant stimulation of respiration of tissue 5 cm from the dead area. Liming the leaves of gladiolus plants did not affect their respiration rate, and did protect them against fluoride damage.

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# THE ISOLATION OF NUCLEI AND OXIDATIVE PARTICLES FROM VIABLE WHEAT EMBRYOS[1,2]

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This paper describes a procedure for the preparation of pure suspensions of cell nuclei from viable wheat embryos. Hitherto, no satisfactory method for the isolation of plant nuclei has been reported although the procedures tested have been those successfully applied to animal tissues. In aqueous media, which are the media commonly used for cell fractionations, plant nuclei break and their contents disperse. In non-aqueous media, although nuclei and nuclear fragments remain coherent, final purification of the nuclear fraction is difficult; even with wheat germ, which is rich in nuclei, it cannot be freed from considerable cytoplasmic contamination (12). One of the contaminants is cytochrome oxidase but the others have not been identified.

A good fractionation procedure for the whole of the plant cell is desirable, but this is unlikely to be achieved until cytoplasmic structures can be morphologically identified in vitro. The procedure here described is therefore aimed largely at the isolation of nuclei, for which wheat germ is obviously the material of choice. Furthermore, the limitations of milled wheat germ for physiological studies do not apply to viable wheat embryos which can now be isolated in quantity (6).

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## METHODS

The following procedure was finally adopted for isolation of the nuclei. Unless otherwise indicated, all steps were performed within the range of 0 to 5° C. Solutions were made up to concentration at approximately 20° C and then chilled for use. For microscopic examination, a drop of tissue suspension was mixed on a slide with a drop of iodine solution which stained the cell fractions quickly and effectively. Starch granules were easily distinguished by their blue coloration.

1. (a) About 10 gm of viable embryos are collected just after being floated off the 2 M sucrose solution (6), and without further washing are cut into sections of 25 microns thickness on a freezing microtome. The common type of microtome may be modified to accommodate 5 gm of tissue at a time by enlarging the yoke and replacing the 1-inch head with a 2-inch one. Or, (b) about 10 gm of embryos obtained by flotation on a cyclohexane-carbon tetrachloride mixture (6) are ground in an agate mortar. The use of smooth agate rather than rough porcelain is advised because the objective is more to spread than to grind the tissue. The powder so obtained consists largely of intact, but separated cells.

2. The embryo sections or powder are suspended in 100 ml of 2 M sucrose which is 0.001 M with respect to $CaCl_2$. The suspension is ground in a stainless steel blendor ("Omnimixer") for 15 minutes at a

setting of 35 volts. The metal container is immersed in an ice-bath during this interval.

3. The homogenate is filtered successively through a single thickness of muslin, a layer of 10 XX mill silk, and two thicknesses of flannelette which are first wetted with 2 M sucrose. The filtrate is spun in an anglehead centrifuge for 15 minutes at 12,000 × g (centrifugal forces are calculated for the maximum radius of the head). The supernatant, if desired, is set aside for the isolation of oxidative particles; the residue is suspended by means of a rubber policeman in a sucrose solution containing 205 gm of sucrose per 100 gm of water. Although supersaturated this solution did not deposit crystals after standing at 0° C for several days. The suspension is centrifuged at 56,000 × g for 15 minutes. The residue contains starch and cell wall debris, and is discarded; the supernatant is diluted to approximately 2 M concentration with one half volume of 0.5 M sucrose, and is centrifuged at 5,400 × g for 30 minutes. The use of water for this dilution step is not recommended because the nuclei tend to hydrate and swell.

The residue, which consists mainly of nuclei, is suspended in 2 M sucrose and centrifuged at 5,400 × g for 30 minutes to remove small particles. This last step is repeated once to complete the procedure for nuclear isolation.

Oxidative Particles: If the solution previously set aside is centrifuged at 140,000 × g for 15 minutes, a residue consisting of a few nuclei and a variety of particles is obtained; this fraction shows moderate oxidative activity. If the supernatant is then diluted to 1 M with water and centrifuged at 36,000 × g for 20 minutes a very active oxidase fraction sediments.

Nucleic Acids: The Schmidt-Thanhauser-Schneider method was followed for the extraction and separation of RNA and DNA (13). The Ogur-Rosen procedure (7) gave similar results although it was not used in calculating the values reported here. Neither method was satisfactory for an accurate determination of RNA in the tissue fractions. Interfering substances in the RNA extract rendered useless not only ultraviolet absorption measurements, but also ribose determinations by the orcinol method of Bruckner (2). Thus, there was no good means of checking on the specificity of the phosphorus determinations. P/N ratios of the RNA extracts were approximately 0.05, one-twelfth the expected value for pure RNA, indicating a strong contamination by other nitrogenous compounds. The analyses for DNA, on the other hand, appeared to be satisfactory. The P/N ratios of the nuclear extracts were of the order of 0.5. Although UV spectra were too impure for quantitative comparisons, desoxyribose and phosphorus measurements agreed closely. The phosphorus contents of the extracted DNA as calculated from desoxyribose measurements were 8.2 % for the whole tissue and 8.9 % for the nuclei. The calf thymus DNA used for standardizing the desoxyribose test had a P content of 9.2 %. The modified diphenylamine procedure of Burton (3) was followed for desoxyribose determinations and the effects of non-specific absorption at the 600 mμ peak were eliminated by means of a second optical density reading at 540 mμ, the difference between these being used for construction of the calibration curve. No DNA was found in the RNA extracts.

Cytochrome Oxidase: This was measured spectrophotometrically by the procedure of Hogeboom and Schneider (5). The cuvette chamber was maintained at about 25° C by means of a cooling attachment. Readings were taken every minute and rates were calculated from the first three minutes, the reaction being linear for that interval. The amounts of tissue used were such that the activities measured were directly proportional to concentration. For the oxidized and reduced forms of cytochrome c, extinction coefficients of 0.9 and $2.87 \times 10^{-7}$ cm² per mole, respectively, were used to express the activities in terms of micromoles of ferrocytochrome c oxidized.

## Results and Discussion

Isolation of Nuclei: There are three critical steps in the procedure outlined, all of which are important in preserving the intactness of the cell-free nuclei: fragmentation of the embryos, suspension of the fragments in concentrated solutions of sucrose, and addition of $CaCl_2$ to the blending medium. It is of considerable advantage that the embryo is a highly desiccated tissue so that hypertonic solutions do not shrink or distort the cells.

A partial fragmentation of the embryos prior to blending is necessary for a good yield of nuclei. Intact embryos blended at a setting of 35 volts do not release nuclei at all. Large fragments, obtained by grinding embryos with dry ice in a Wiley mill, release relatively few nuclei after 3 or 60 minutes of blending. Sectioned embryos yield a maximum number of free nuclei after 15 minutes, and although a large number of unbroken cells remain in the embryo sections no additional nuclei are released even after an hour of blending. It is important to note that the techniques of fragmentation keep artifacts to a minimum. Apart

Table I

The Effect of Calcium Ion in Preventing Rupture of Nuclei in Sucrose Media

| M conc. $Ca^{++}$ in 2 M sucrose | No. nuclei/gm starting material |
|---|---|
| 0.0 | 22,000 ± 15 % |
| 0.001 | 400,150 |
| 0.01 | 357,900 |

Fifty grams of commercial germ were washed free of starch by stirring in a 2 M sucrose medium (with or without calcium) and filtering through flannelette. The residue was ground in an "Omnimixer" for 30 min at 30 volts, and filtered to remove unbroken tissue. The nuclei obtained by centrifugation were suspended in a known volume of sucrose and counted by means of a hemocytometer. In this procedure less than 5 % of the tissue cells were broken, but the same differences with respect to calcium were readily apparent in preparations using the procedure described under Methods.

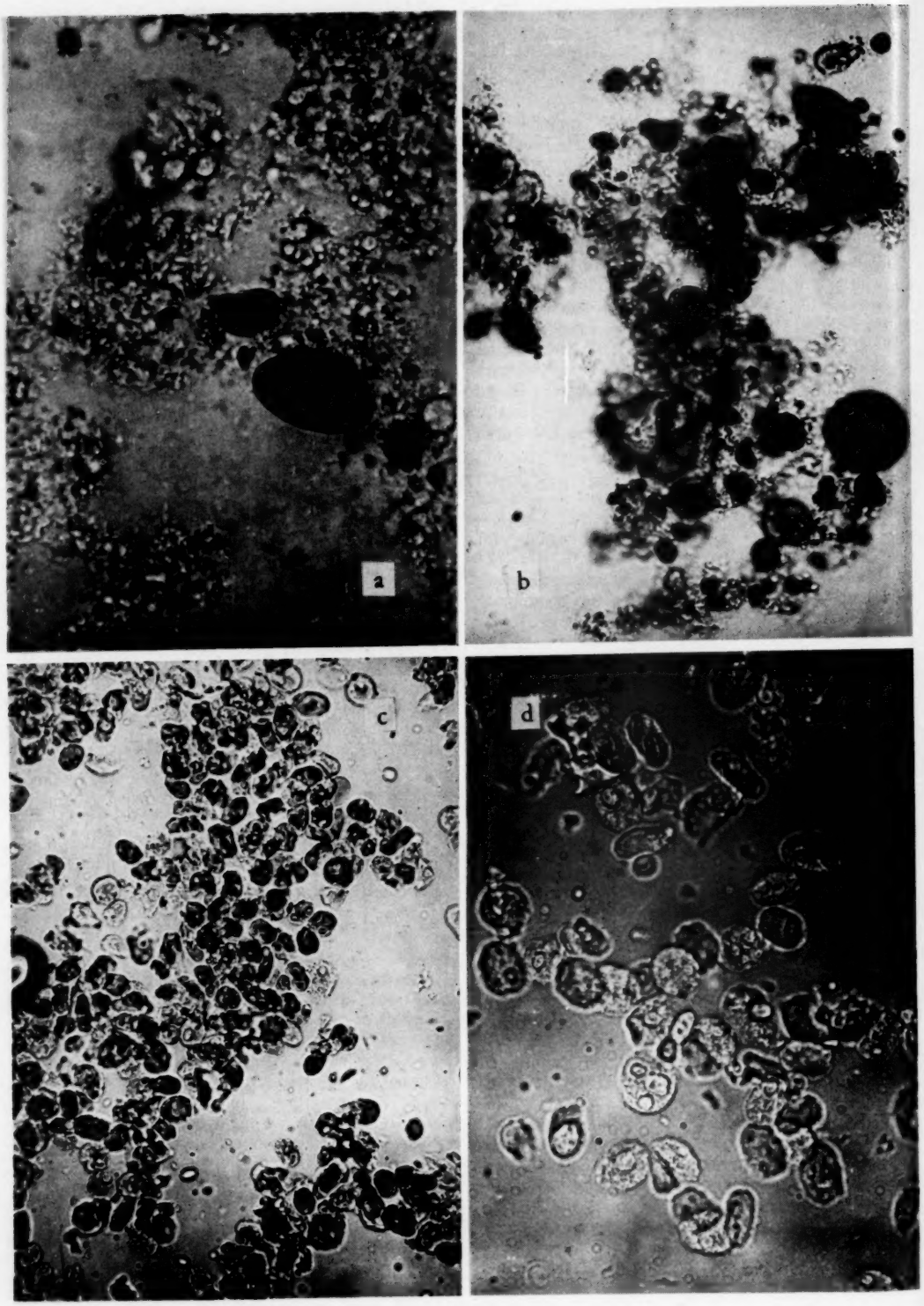
a
b
c
d

TABLE II

NUCLEIC ACID CONTENT OF WHEAT EMBRYOS AND OF ISOLATED NUCLEI

| FRACTION | ACID-INSOLUBLE N IN SAMPLE | TOTAL P | RNA-P | DNA-P | RNA-P / N | DNA-P / N |
|---|---|---|---|---|---|---|
| | *mg* | *mg* | *mg* | *mg* | % | % |
| Whole tissue | 103 | 10.2 | 7.4 | 1.6 | 7.2 | 1.55 |
| Purified nuclei | 3.6 | 0.58 | 0.1 | 0.38 | 2.8 | 10.55 |

The values listed are based on two separate preparations and duplicate measurements of each. The average amount of tissue used for each series of analysis is indicated in the first column as total acid-insoluble N.

from mechanical breakage, the procedures followed in preparing the tissues for disintegration do not impair the viability of the cells. Embryos kept frozen in 2 M sucrose for twenty minutes and then thawed, or those floated off cyclohexane-carbon tetrachloride mixture, all germinated when sprinkled on agar plates. Of considerable interest is the fact that organic solvents, although they do not affect germination of the embryos, do affect their physical quality. Embryos isolated in this way are easily ground into a powder consisting largely of separate cells, whereas the cells from sucrose-floated germ disintegrated when similarly ground. The advantage of grinding over sectioning is that the yield of nuclei is increased some 5-fold; the disadvantage is that a small percentage of whole cells collects with the nuclei throughout the isolation.

The high concentration of sucrose serves two purposes. First, it helps to prevent rupture of the free nuclei. If, for example, the 2 M sucrose used in blending is replaced with 0.25 M, very few, if any, intact nuclei remain. Second, it is possible to separate cell debris from the nuclei by use of supersaturated solutions of sucrose. The effectiveness of such separation depends to some extent on the degree of hydration of the embryos prior to fragmentation. Storage of the embryos over $H_2SO_4$ (specific gravity = 1.20) at 4° C for 24 hours has proved satisfactory as a means of assuring a good yield of nuclei.

Calcium chloride is introduced to reduce further the breakage of nuclei. Low concentrations of this salt have been used to prevent clumping of rat liver nuclei (8), but the phenomenon has not been observed in these nuclei, perhaps because of their lower DNA concentration. The effectiveness of $CaCl_2$ in reducing fragmentation of wheat nuclei is demonstrated in table I.

The product obtained by the procedure outlined is illustrated in the accompanying figure. If embryo sections are used as the starting material, about 10 to 20 % of the DNA present in the flannelette filtrate is recovered in the final nuclear preparation. The percentage recovery of intact nuclei is much greater, however, since a large fraction of the DNA present in the filtrate is derived from nuclei which have been broken and dispersed in the course of sectioning. Clearly, the method is unsatisfactory for a quantitative fractionation of the germ, but qualitatively, the method is good. The only contaminants present in the nuclear preparation are small particles. The extent of this contamination may be judged from figure 1 d. Occasional particles are starch and these can be spotted by their reaction with iodine. Others may be oxidative particles, but it will be shown that these can constitute no more than 3 % of the total nitrogen of the preparation.

NUCLEIC ACID CONTENT: A summary of analytical values is given in table II. From the percentage (DNA-P)/N in the nuclei it may be calculated that about 6 to 7 % of the total weight of nucleic acid plus protein is DNA (taking nucleic acid to be 10 % P and 16 % N and protein to be 16 % N). This value is small compared to those obtained for many species of mammalian nuclei isolated in non-aqueous media (1), but it is twice that previously obtained for wheat germ (12). The discrepancy between the two preparations of wheat nuclei may in part be accounted for by the probable loss of soluble protein from the sucrose-isolated nuclei and by their higher degree of purity. Obviously, a more certain comparison would require a better procedure for non-aqueous isolation of wheat nuclei. Yet, despite the differences between the preparations, the results agree on the point that compared with mammalian nuclei, wheat germ nuclei have a low DNA and a high protein content. To what extent this is a characteristic of plant nuclei remains to be explored.

The proportion of embryo N which is nuclear may be calculated from the percentages of DNA-P in whole tissue and nuclei, respectively. (DNA is restricted to the nuclei so that the concentration measured in the whole tissue may be regarded as a dilution of the nuclei by non-nuclear nitrogen.) Thus calculated, nuclei account for 15 % of embryo N, a very low figure compared to the 50 % reported for the non-aqueous preparation (12). A loss of soluble pro-

FIG. 1. Stages in the isolation of nuclei from viable wheat germ tissue. The preparations are all contained in 2 M sucrose to which a drop of conc iodine solution was added in order to stain the cell fragments. a (*upper, left*). Initial homogenate (×540). b (*upper, right*). First sediment obtained by centrifuging homogenate at 12,000×g for 15 min (×294). c (*lower, left*). Final nuclear preparation (×215). d (*lower, right*). Nuclear preparation (×500).

tein from animal nuclei isolated in sucrose media has been established (11), and wheat nuclei probably behave in much the same way. Such loss of protein and the higher degree of purity of the sucrose nuclei are undoubtedly two of the reasons for the difference. The improved procedure for DNA determination is probably another. To judge frrom the cytological picture, the real proportion of nuclear nitrogen is somewhere between 15 and 50 %.

Ribonucleic acid measurements indicate that the bulk of embryo RNA lies in the cytoplasm. Per mg N, RNA-P is 2.5 times as concentrated in the whole tissue as in the nuclei, and since the latter account for only 15 % of the tissue nitrogen, nuclear RNA may be estimated as 6 % of the total. Within the nucleus 20 % of the nucleic acid is of the ribose form. Since 50 % of the nucleic acid of non-aqueous nuclei of wheat was found to be RNA (12), the possibility arose that a soluble "ribonucleic acid" might have been leached out of the sucrose nuclei. A preliminary check on this possibility was performed in the following way: The supernatant remaining in 2 M sucrose after sedimentation of the nuclei was diluted to 1 M with water and the pH adjusted to 5.0 with 5 M acetate buffer. Without acidification, a slightly turbid supernatant remained even after centrifugation at 140,000 × g. Some soluble "RNA" might have been precipitated in this step but acidification appeared to be preferable to analyzing for RNA in the presence of microsome-like particles. The acidified suspension could be clarified by centrifugation at 12,000 × g for 30 minutes. It was then diluted with ¼ volume of 25 % TCA (1.8 M) and the resultant precipitate extracted by the Schmidt-Thanhauser-Schneider procedure (13). The RNA content of the extract was found to be approximately 1 % of the total RNA of the tissue. Since nuclear RNA is about 6 % of the total, the whole of the RNA measured in the supernatant would, if derived from the nuclei, increase the concentration of nuclear RNA by ⅙, raising it to 23 % of the total nucleic acid of the nucleus. This value would still be far removed from the 50 % found in the non-aqueous nuclei. The discrepancy needs to be resolved by further experiment, and until then, the earlier conclusion that wheat germ nuclei have a very high content of RNA along with a high nitrogen content must be regarded as suspect.

CYTOCHROME OXIDASE: There are three principal points of interest in connection with the oxidase measurements listed in table III.

First, the activity of the whole tissue homogenate is about nine times the activity of a number of samples of commercial germ tested by the same spectrophotometric procedure. In his original work with commercial germ, Goddard (4), using a manometric technique, obtained an oxygen uptake of 240 $\mu$l/hr × 50 mg of germ. If the germ is estimated as 5 % protein N, then this value may be rewritten as 8 microatoms of oxygen/hr × mg N or 0.8 micromoles of cytochrome c/3 min × mg N. This is one-eighth the value obtained with viable wheat embryos.

Second, the specific activity of the isolated oxidase particles is higher than that generally recorded for plant preparations. A recent measurement of cytochrome oxidase activity in particles prepared from germinating peas (9) gave an E of 78/mg N × 3 min, a value lower than that obtained for homogenates of whole embryo. Stafford (10), using manometric techniques for measurement of oxidase activity in particles of pea seedlings, found an uptake of 260 $\mu$l $O_2$/hr × mg protein. This is equivalent to about 14 micromoles of cytochrome c oxidized/3 min × mg protein N, which is one-third of that obtained for the wheat germ particles. The closest in oxidase activity to the wheat particles are the highly purified preparations of rat liver mitochondria which, measured under similar conditions, oxidize 56.7 micromoles of reduced cytochrome c/3 min × mg N (5). Thus Goddard's speculation that the oxidase of the embryo is fully developed even though respiration rises markedly with germination is consistent with the evidence here obtained for highly active oxidase particles. Whether the high activity of the wheat germ particles becomes higher yet in the germinating embryo cannot be determined in the absence of a morphological criterion of purity of particulate preparations.

Third, wheat germ nuclei, like isolated nuclei from a diversity of animal tissues, do not possess cytochrome oxidase activity. The conclusion is based on the decreasing oxidase activity per mg protein N with increasing purification of the nuclei, and on the very high activity of the oxidase particles which can best account for the residual activity in the purified nuclear preparation.

TABLE III

CYTOCHROME OXIDASE ACTIVITIES OF VIABLE WHEAT GERM PREPARATIONS

| FRACTION | $-\Delta E_{550}$/MG N | MICROMOLES CYT. C OXIDIZED IN 3 MIN/MG N |
|---|---|---|
| Whole tissue homogenized | 132 | 6.2 |
| Whole tissue less nuclear sediment | 192 | 9.7 |
| First particulate sediment | 225 | 11.5 |
| Second particulate sediment | 960 | 48.5 |
| Nuclear preparation | 48 | 2.4 |
| Purified nuclear preparation | 27 | 1.4 |

The changes in extinction values ($\log_{10} I_o/I$) are calculated for a 3-min period for 3 ml of reaction medium with a light path of 1 cm. Four or more measurements were made for each tissue fraction. Conditions of reaction were as described under Methods.

## SUMMARY

A method has been described for the isolation and purification of nuclei from viable wheat embryos. A highly active preparation of oxidative particles has also been obtained. Tissue and nuclei were analyzed for nucleic acids and the significance of the measurements discussed.

Comparable preparations of isolated nuclei have since been obtained from viable embryos of oats, barley and rye by means of the procedure described here.

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# INDUCTIVE CONTROL OF INDOLEACETIC ACID OXIDASE ACTIVITY BY RED AND NEAR INFRARED LIGHT[1,2]


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Many plant processes, including seed germination, photoperiodic flower induction, and the development of stems and leaves, can be affected by brief exposures to low energies of light. Action spectra for such light effects resemble each other closely, with red light (ca 660 mμ) showing maximum effectiveness. In addition, the effects of red light can usually be prevented by exposure to near infrared[3] radiation (ca 730 mμ) sufficiently soon after red light treatment. These characteristics have led to the belief that one photoreaction and pigment system may control widely diverse processes (2, 3, 9, 19). Although the relation between this photoreaction and a biochemical system which might control plant growth is unknown, there is considerable evidence that auxin metabolism may be involved (5, 7, 11, 12, 13). Since the indoleacetic acid (IAA) oxidase system may be a controlling factor in auxin metabolism (6), its relation to red light action has been investigated in the present work. The results indicate that the IAA oxidase activity of certain tissues is controlled by a low energy photoreaction apparently typical of those controlling photoperiodism and the other processes mentioned.

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[2] Research supported in part by the National Science Foundation under Grant NSF G-2009.

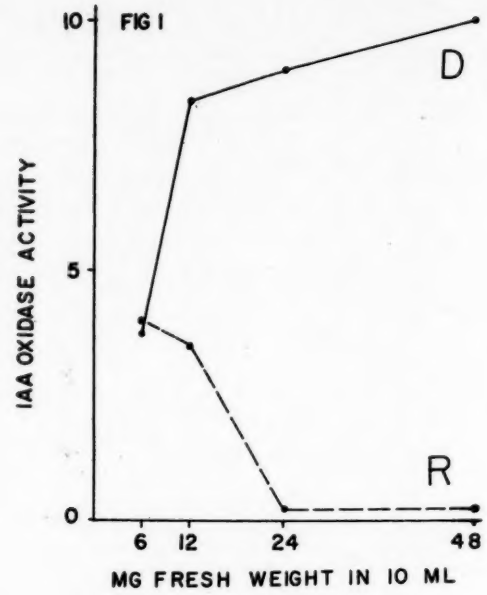
[3] Wassink and Stolwijk (19) have pointed out why "infrared" is preferable to the term "far-red" to designate this region of the spectrum.

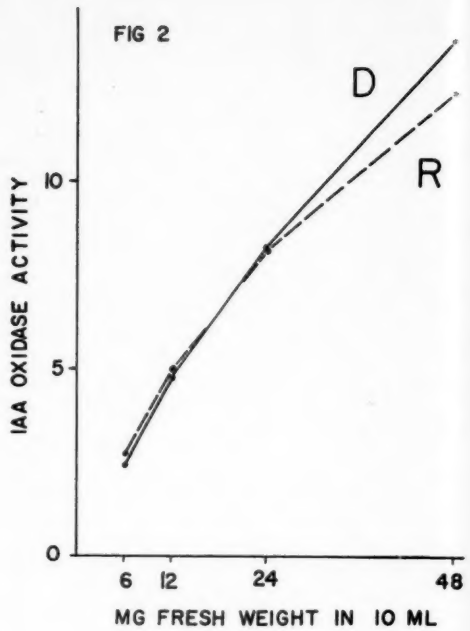
## General Procedures

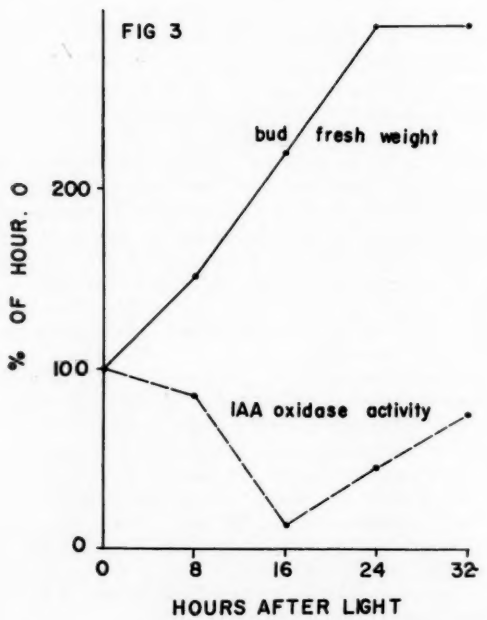
Most of the experiments were conducted with peas (*Pisum sativum* L. var. Alaska) obtained from Associated Seed Growers, Inc., New Haven, Connecticut. The seedlings were grown in vermiculite for 7 days at 26° C in darkness, with occasional dim green light during handling. Some were also exposed to 2 to 2500 kiloergs/cm² of red light during a brief period 5 minutes to 40 hours before harvest. Red light was supplied by Sylvania red fluorescent tubes with maximum emission at 640 mμ and none below 580 mμ. Energy measurements were made with a phototube light meter whose construction and calibration is described elsewhere (5).

Tissues harvested for IAA oxidase assays were weighed, placed immediately at −16° C, and stored at that temperature until use (1 to 10 days) with little or no resultant loss in activity. For assay, the frozen tissue was rapidly reduced to a powder with a chilled mortar and pestle and ground further with 0.02 M phosphate buffer, pH 6.1. The preparation was centrifuged in the cold to remove cellular debris and the clear supernatant extract made to volume and assayed. Usually 600 mg fresh weight of tissue were made to a final volume of 25 ml.

The assay used, described in more detail elsewhere (10), consists of colorimetric determinations of residual IAA in reaction mixtures incubated at 26° C. Each 10-ml reaction mixture contained 0.5 to 5 ml extract, 2 ml 0.01 M phosphate buffer, pH 6.1, and a



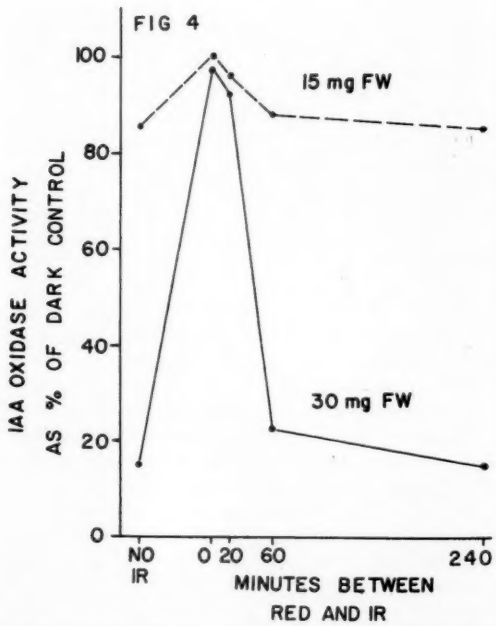




FIG. 1 (*upper, left*). IAA oxidase activity (μgm/ml IAA destroyed in 60 min in a 10-ml reaction mixture started at 35 μgm/ml) as a function of crude extract concentration representing the indicated tissue fresh wts. D, extract from buds of dark-grown plants. R, extract from buds of plants exposed to 750 kiloergs/cm$^2$ red light 16 hrs before harvest.

starting IAA concentration of 35 μgm/ml ($2 \times 10^{-4}$ M). Either $10^{-4}$ M 2,4-dichlorophenol (DCP) or $10^{-4}$ M $MnCl_2$ was usually present as well, as cofactor for the enzyme system (7, 10). Activity without either Mn or DCP was too low for reliably significant readings, and was rarely tested after the preliminary experiments. Activity was expressed as μgm/ml IAA destroyed in 20 or 60 minutes, either on a fresh weight basis or as specific activity (6) on a protein nitrogen basis.

## Experimental

Preliminary—IAA Oxidase Inhibition in Buds: Experiments were conducted to see what tissues, if any, would exhibit changed IAA oxidase activity after exposure to red light. The test plants were exposed to a total energy of 750 kiloergs/cm² during one hour and returned to darkness. Terminal buds and apical 1-cm sections of the terminal internode were harvested 16 to 18 hours after the light treatment, and their activities compared with that of totally dark-grown controls harvested at the same time. The 16 to 18 hour interval between illumination and harvest was chosen since it is the optimal time for expression of the changed auxin sensitivity in the internode section test (5). When assays were conducted at extract levels representing 50 mg fresh weight in each 10-ml reaction mixture, in the presence of $10^{-4}$ M Mn or DCP, the IAA oxidase activity of buds of red-illuminated plants was depressed to 5 to 15 % of that of the dark control buds on a fresh weight basis. Specific activities maintained the same relationships, since the ratio of protein nitrogen to fresh weight did not change significantly following illumination. An 85 to 95 % inhibition of activity in the buds could thus be attributed to the red light treatment. This inhibition was observed in assays either with DCP or Mn alone, or with both present in various proportions. Since corresponding experiments with the internode sections failed to show differences of any magnitude or consistency between dark-grown and red-illuminated tissues, bud tissues only were used in most of the subsequent experiments.

Extract Dilution—Evidence of an Inhibitor: For a more detailed comparison of the activities of extracts from red-illuminated and dark-grown buds, the effect of extract concentration in the reaction mixture was studied. The results of a typical experiment are shown in figure 1. Amounts of extract representing 6, 12, 24 or 48 mg fresh weight were used in standard 10-ml reaction mixtures, all with $10^{-4}$ M DCP. At the higher extract levels, the inhibitory effect of prior red light treatment was very evident. At lower levels, however, both activities approached the same value. The fact that the activity of extract from red-illuminated buds was so markedly increased by dilution suggested the presence of an inhibitor. To test this possibility, attempts were made to increase the activity of the extract by dialysis.

Centrifuged extracts of both red-illuminated and dark-grown buds were prepared, 600 mg fresh weight being made up to 25 ml of extract. One 10-ml portion of each was dialyzed in a cellophane membrane against 6 l of 0.02 M phosphate buffer, pH 6.1, at ~ 2° C. Another 10-ml portion of each was stored in a cellophane membrane at the same temperature as a control. After 24 hours, the extracts were assayed at various concentrations in the presence of $10^{-4}$ M DCP. The activities of the dialyzed extracts are shown in figure 2. The undialyzed controls supplied the data used in figure 1. Two effects of dialysis are evident from a comparison of the figures. The activities of both types of extract were greatly increased, and the difference between them was almost entirely removed. Apparently both extracts contained a dialyzable inhibitor, or inhibitors, but with a higher level present in that from the red-illuminated buds.

In order to test this hypothesis further, dialysates from both types of extract were assayed for inhibitory effects. Extracts of red-illuminated and dark-grown buds were prepared as usual. Ten ml of each was placed in a cellophane dialysis membrane and dialyzed against 10 ml of buffer in a large test tube at ~ 2° C for 24 hours with vigorous shaking. The extracts were discarded, and various amounts of the buffered dialysates added to standard reaction mixtures containing $10^{-4}$ M DCP and a highly active enzyme preparation from internode tissue. The dialysates from extracts of red-illuminated buds were at least 4 times as inhibitory as those from dark controls, on a fresh weight basis. Similar results were obtained by adding boiled extracts instead of dialysates as sources of inhibitor.

The simplest conclusion is that the inhibition of activity induced by red light treatment is due entirely to increased levels of a dialyzable, heat-stable inhibitor (or inhibitors). In the absence of any direct test for the inhibitor, however, further experiments were conducted by comparing bud extracts from variously-treated plants at fresh weight levels of 24 or 30 mg per 10-ml reaction mixture, and always in the presence of $10^{-4}$ M DCP.

Timing of Red Light Treatment: In the preceding experiments, 16 to 18 hours was interposed between red light exposure and harvest, on the assumption that a maximum effect would result. The

Fig. 2 (*upper, right*). Like figure 1, except with dialyzed extracts, and activity measured over 20 min.

Fig. 3 (*lower, left*). IAA oxidase activity and bud fresh weight at various times after red light treatment (225 kiloergs/cm²). Activity measured as in figure 1, with 12 mg FW per reaction mixture; value for hour 0, 13.4. Average bud fresh weight, hour 0, 6.1 mg.

Fig. 4 (*lower, right*). The effect of time elapsed (in darkness) between red and near infrared (IR) treatment on the subsequent IAA oxidase activity. Red light 75 kiloergs/cm² given 16 hrs before harvest. IR, 4 min. Dark control activity, (as in fig 1) for 30 and 15 mg fresh weight levels, 17.0 and 16.3, respectively.

effects of red light timing were next studied directly by harvesting tissues immediately, or 4, 8, 16, 24, 32 or 40 hours after 225 kiloerg/cm² light treatment (15 min). Such experiments can be performed in two ways: All plants can be illuminated at the same time, with harvests at various times afterwards; this has the advantage of exposing all plants at the same developmental stage, but the disadvantage of harvesting plants of dissimilar ages. Conversely, groups of plants can be illuminated at different times and all harvested at the same time, with the opposite considerations. Fortunately, both types of experiments agreed. Results of an experiment of the first kind described are shown in figure 3. IAA oxidase activity and average bud fresh weight are presented for comparison as percentages of the zero time value, i.e., immediate harvest. Data for the dark controls remained substantially constant during the experimental period, and activity at zero time did not differ from the dark controls. Of the times chosen, illumination 16 hours before harvest produced the maximum inhibition of IAA oxidase in the buds. Tissues harvested as long as 32 hours after light (40 in other experiments) still showed lower activity than the dark controls.

The bud weight data in figure 3 are of particular interest. While the minimum IAA oxidase activity was always obtained about 16 hours after illumination in such experiments, bud size continued to increase for at least 24 hours, and in some experiments 32 or 40 hours, after illumination. In more general terms, the maximum effect of light was expressed sooner in the inhibitor level than in bud weight, suggesting that the process controlling inhibitor level may be more closely linked to the photoreaction. While this conclusion may not be justified, figure 3 at least indicates clearly that the results reported here are not merely consequences of assaying morphologically different tissues, as they might be if a simple inverse relation existed between bud size and IAA oxidase activity. In further experiments, inhibition has been obtained as little as three hours after illumination, before any weight increase or gross morphological change could be detected.

RED LIGHT DOSAGE: In order to determine the energy required to produce a measurable inhibition, groups of plants were exposed to 2, 20 or 200 kiloergs /cm² within a 15-minute period 16 hours before harvest. The activities of bud extracts were then compared with that of the dark controls at fresh weight levels of 24 and 12 mg per reaction mixture. Measured at the 24-mg level, all dosages produced about 90 % inhibition on both fresh weight and specific activity bases. Measured at the 12-mg level, the corresponding inhibitions were 2, 11 and 48 %. While the effects of dilution indicate the impossibility of obtaining quantitative data without a direct assay for the inhibitor in question, it is still evident that the effects of as little as 2 kiloergs/cm² of red light are easily detectable under the proper conditions.

NEAR INFRARED REVERSIBILITY: A 1000-watt incandescent light, separated from the plants by 10 cm of water and 4 thicknesses each of dark red and dark blue cellophane, was used as the source of infrared radiation (2, 3). The distance from the filament to the plant tops was approximately 58 cm. Preliminary experiments showed that 30 seconds of such irradiation was sufficient to cause over 90 % reversal of the effects of 30 to 75 kiloergs/cm² red light, if given immediately after the red light treatment. When several rapid alternations of red and infrared (IR) were carried out, the final effect depended upon the last radiation given. In the data presented in table I, the activities of buds from plants receiving IR alone, or red followed by IR, or red-IR-red-IR, were essentially like the dark controls, while the treatment red-IR-red caused the same inhibition as red alone.

Since 30 seconds of IR caused essentially complete reversal, the intensity of the source was reduced to less than 1/3 with a variable-voltage transformer in order to obtain dose-response data. Such data, however, like those with red light, are rendered difficult to interpret by the effects of enzyme dilution in the assay. In a typical experiment, all radiations were given about 16 hours before harvest, and the buds were assayed at 30 and 15 mg FW per 10-ml reaction mixture (with $10^{-4}$ M DCP). Seventy-five kiloergs/cm² red light (5 minutes) inhibited activity 96 % as assayed at the higher fresh weight level, and 40 % at the lower. Ninety seconds of (reduced intensity) IR given immediately after the red was sufficient to remove inhibition as assayed at the lower level, while 4 to 5 minutes was required at the higher.

In spite of such complications it was possible to obtain fairly precise data on the length of time during which the red light effect remains reversible by IR. For the experiment summarized in figure 4, all plants (except the dark controls) were first exposed to 75 kiloergs/cm² of red light (5 min). Some were exposed to 4 min of IR immediately afterwards, while others received IR 20 minutes, one hour, or four hours after the end of the red light. Bud tissues were har-

TABLE I

IAA OXIDASE ACTIVITY OF PEA BUDS 16 HOURS AFTER EXPOSURE OF INTACT PLANTS TO RED AND NEAR INFRARED (IR) RADIATION IN SEQUENCE*

| TREATMENT | IAA OXIDASE ACTIVITY** | INHIBITION, % |
|---|---|---|
| Dark controls | 8.3 | (0) |
| IR only | 8.0 | 4 |
| Red only | 0.7 | 92 |
| Red-IR | 7.4 | 11 |
| Red-IR-Red | 1.0 | 88 |
| Red-IR-Red-IR | 7.6 | 8 |

* Red light exposure 2 min, total energy 30 kiloergs/cm². IR exposure 2 minutes (high intensity—see text). Less than 1 min elapsed between successive exposures.

** Activity measured as μgm/ml IAA destroyed in 20 minutes. Each 10-ml reaction mixture contained 35 μgm/ml IAA at the start, $10^{-4}$ M DCP, and bud extract equivalent to 24 mg fresh wt.

vested 16 hours after the initial (red) radiation and assayed as usual at 30 and 15 mg fresh weight levels. It is evident from figure 4 that, at 26° C, the red light effect is only very slightly reversible by IR given more than 1 hour afterwards. This is true even according to the assay at the lower fresh weight level, in which the apparent inhibition due to red light was small.

PEA INTERNODES AND EXCISED BUDS: Although no effect of red light on the IAA oxidase activity of internode tissue was observed in the preliminary experiments, the results with buds suggested that a wide range of extract concentrations should be tried before reaching this conclusion. Accordingly, the effect of 750 kiloergs/cm$^2$ given 16 hours before harvest was re-examined. Apical 1-cm portions of the 3rd (terminal) internodes from illuminated and control plants were compared at fresh weight levels from 12.5 to 200 mg per reaction mixture, with $10^{-4}$ M DCP. The initial observation was confirmed.

The effect of red light on the IAA oxidase activity of excised buds was also investigated. Six hundred-mg lots of buds were excised from 6-day-old dark-grown peas and placed in beakers with 0.02 M phosphate buffer, pH 6.1. Some were then exposed to 750 kiloergs/cm$^2$ red light, the others retained in darkness. The tissues were recovered after 16 hours, and later assayed as usual. While activity declined slightly as a result of excision, no effect of the red light could be detected. Neither the inclusion in the medium of 2 % sucrose plus 0.05 M $KNO_3$, nor of cotyledons from germinating peas, would confer any light-sensitivity in subsequent experiments.

BEAN AND LUPINE BUDS: Other plant materials were cursorily investigated for two reasons. It seemed desirable to see whether the results obtained with peas might be generally applicable to other plants. It would also be extremely useful for further experiments to obtain the response in excised tissues which might then be exposed to various substances and rapid changes in temperature.

The entire hypocotyl was removed from 2-day-old Black Valentine Bean (*Phaseolus vulgaris* L.) seedlings grown in darkness at 26° C, leaving only the two cotyledons and the enclosed plumule. One cotyledon was also removed, and the remaining tissue placed, plumule upwards, in a Petri dish with buffer. Twenty-five such plumules with cotyledons were used for each experimental treatment with radiation or darkness. Eighteen hours after radiation, the plumules (mainly young leaf tissue) were harvested for the usual assays.

In these experiments, 75 kiloergs/cm$^2$ of red light (the lowest energy tried) affected IAA oxidase activity precisely as in the intact pea buds. Attempts at infrared reversal, however, were completely unsuccessful over a wide dosage range. IR given alone acted in the same way as red light—it induced a great decrease in IAA oxidase activity. When a different IR filter system was used, consisting of a Corning HR Red-Purple-Ultra filter (15) and two thicknesses of red cellophane, the same results were still obtained.

An experiment was performed with buds of 5-day-old seedlings of *Lupinus alba* handled in the same way as the beans. Activity of the extract was extremely high compared with peas or beans, even in the absence of DCP or added manganese, and no evidence of an inhibitor could be detected in dark-grown tissue. Exposure to 750 kiloergs/cm$^2$ red light had no effect on activity.

## DISCUSSION

The major experimental findings can be briefly summarized. The IAA oxidase activity of intact pea buds, as assayed in extracts, was inductively inhibited by total energies of red light as low as 2 kiloergs/cm$^2$ given to the intact plants at some time before harvest. The inhibition was reversible by near infrared radiation given immediately after the red light. The inhibition appears to be due entirely to increased levels of a thermostable, dialyzable inhibitor (or inhibitors) originally present in dark-grown plants as well.

Tang and Bonner (18) were the first to report that light exposure reduced the IAA oxidase activity of etiolated peas by causing an increase in the level of an inhibitor. In their experiments, inhibitor concentration increased proportionally to the number of hours of diffuse daylight given. The present results differ chiefly in showing that IAA oxidase inhibition can be controlled by low energies of photoperiodically active radiations.

A number of reports have appeared in which the auxin metabolism of etiolated or semi-etiolated material was modified by red light and near infrared treatment. Kent and Gortner (11) found that the auxin sensitivity of the split pea epicotyl test was greatly increased by exposure of the plants to red light 32 hours before use. Galston and Baker (5) showed that the auxin sensitivity of pea internode sections was reduced by exposure to about 2000 kiloergs/cm$^2$ red light 16 to 18 hours before the test. Goldacre et al (7) found the IAA oxidase activity of extracts of entire pea epicotyls, assayed in the absence of added DCP or manganese, to be much higher when plants were grown under continuous red light rather than in darkness. Liverman and Bonner (13) were able to show that the response of Avena coleoptile sections to auxin was promoted by red light; this effect was reversible by infrared, but only when the IR was given in the presence of auxin. Most recently, Klein et al (12) have used red light to decrease the sensitivity of excised hypocotyl hooks of etiolated bean seedlings to IAA.

The use of different materials treated in different ways, and the absence of any experimental attempts to relate one investigation to another, leaves the relationship of all these results completely obscure. This applies with equal force to the present work, and it is possible only to point out certain major differences between it and the reports with which it might be compared. The total light energies needed here were lower by a factor of 1000 than those employed in (5) and (7). The materials tested, the phenomena exam-

ined, or both, were different from those in any of the preceding investigations. No IR reversals were attempted in (5) (7) or (11). In view of this situation, any attempt to discuss the present work in terms of that cited would be sheer speculation. It will be more fruitful to consider the possible physiological significance of the results presented, and also whether they afford any further insight into the red-infrared response.

Several points suggest that the red light induced increase in inhibitor may be of physiological significance. In itself, the inductive nature of the phenomenon is worth noting together with the fact that the maximum inhibition precedes maximum bud weight attained. The extreme sensitivity of bud (leaf) tissue as compared with the internode is of interest since Parker et al (14) reported that the internode response of etiolated peas was relatively slight, and irregular, compared to that of the leaves. The low light intensities needed also suggest a close relationship to deetiolation (2, 14). No attempt was made to determine the most effective region in the red, and the fluorescent source emitted all wavelengths between 580 and about 710 $m\mu$, so it is probable that considerably less than 2 kiloergs/cm$^2$ would be effective at the most active wavelength. Finally, the rapid "escape from photochemical control," after which the effect of red light is no longer reversible by infrared, is similar to that obtained by Downs (3) in the photoperiodic induction of Xanthium and Biloxi soybean.

On the assumption that the results of in vitro IAA oxidase assays are representative of in vivo activities, and on the further assumption that bud growth in the dark is limited by endogenous auxin level, the data presented might be used to explain the increased bud growth induced by red light. The first assumption, however, is debatable. Although there is a considerable body of literature on natural IAA destruction, only two reports have been primarily concerned with its physiological significance. While Galston and Dalberg (6) obtained evidence which related increased IAA oxidase to tissue ageing and cessation of growth in peas, the results of Briggs et al (1) call into question the entire concept of using in vitro extract assays as estimates of in vivo activity. In the fern *Osmunda cinnamomea* L, tissues which would transport auxin without destroying it would destroy it rapidly when converted into extracts. In addition, tissues were also found in which IAA would disappear rapidly, but which would not inactivate it as extracts. Some support for the in vivo significance of IAA oxidase has been derived from the observations of Goldacre et al (7) that 2,4-dichlorophenol (DCP), which enhances the IAA oxidase activity of extracts, also enhances the disappearance of IAA from solutions incubated with pea stem sections. This observation was re-examined and confirmed in the course of the present work, but preliminary results also showed that IAA destruction continues at a high rate for some time after removal of the tissues, suggesting that much if not all of the IAA destruction in such a system is due to leakage of enzymatic material from the short (5-mm) sections used. In view of such findings, any direct physiological interpretation of the present results would be indefensible at this time.

The few experiments with bean and lupine tissues are also relevant to considerations of physiological significance. The ease with which a red light effect similar to that on peas could be demonstrated in bean buds contrasts with the complete inability of near infrared to reverse the action of red. This is the more remarkable since the action of red light on hypocotyl hooks of the same bean variety is clearly infrared-reversible (12); in the present experiments, however, IR acted in the same way as red itself. While no satisfactory explanation for this result is at hand, it may be related to the occasional reports (3, 19) that in cases where brief exposures to IR cause reversal of a red light effect, longer exposures may fail to do so. Whatever the mechanism, this apparent reinforcement of red light action by IR appears to be completely dominant in the bean system studied. Since no evidence of inhibitor was found in the etiolated Lupinus buds, the absence of a red light effect on their IAA oxidase activity is not surprising; it is possible that more thorough investigation would modify these results. In summary, it is evident that the results obtained with peas are probably not directly applicable to other plants, even within the same family.

While the simplest explanation for the present results appears to be an increased inhibitor level induced by red light, as stated earlier, it is not necessarily the correct one. Both naturally occurring inhibitors (4, 8) and activators or cofactors (16, 20) for IAA oxidase have been reported, but not identified. In view of the complexity of the IAA oxidase system, and of the uncertainty as to the precise nature and role of its components, alternate explanations of the increased inhibition caused by dialysates from illuminated plants might be proposed. Even assuming the inhibition to be due to increased inhibitor, the mechanism of inhibitor action is not known. Kinetic observations indicate that it acts by introducing an extended lag period. Thus the inhibition values recorded are only approximations of inhibitor level, since it proved impractical to evaluate the length of the lag period for each extract tested.

Whatever the mechanism of the inhibition, it is clear that the effect of the red light on IAA oxidase activity has been on the dialyzable, heat-stable components rather than on the protein fraction. The results represent a clear-cut biochemical change controlled by photoperiodically active radiation. The syntheses of a tomato cuticle pigment (15) and of certain anthocyanins (17) are known to be controlled by red and infrared light. To this group can now be added the unknown "inhibitor." The control of inhibitor level, and of the other compounds mentioned, suggests an approach to the identification of the light-sensitive system. Retracing their origin to the particular stage at which the light-reaction is the controlling factor would represent a real advance in light

physiology. As an initial step, attempts to identify the chemical basis of the inhibition are now in progress.

## Summary

The IAA oxidase activity of buds of etiolated peas, as assayed in extracts with the addition of 2,4-dichlorophenol or manganous ion, was greatly inhibited by 2 to 750 kiloergs/cm$^2$ red light given to the intact plants during a brief period at some time before harvest. Maximum inhibition with a given energy was obtained by illuminating about 16 hours before harvest. The inhibition was reversible by near infrared radiation given immediately after the red light, but not more than one hour afterwards. The activities of extracts from dark-grown and red-illuminated buds could be made the same by dilution, or by dialysis, suggesting that the inhibition may be due entirely to increased levels of a dialyzable inhibitor. The IAA oxidase activity of young internode tissue was insensitive to the low energies used. The results may be of significance in the physiology of de-etiolation and of other processes controlled by red and near infrared radiations.

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# THE EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID ON PATHWAYS OF GLUCOSE CATABOLISM IN HIGHER PLANTS[1,2]

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In a previous communication from this laboratory (8), it was briefly reported that etiolated pea seedlings (cotyledons removed) treated with 2,4-dichlorophenoxyacetic acid (2,4-D) were able to maintain a higher rate of respiration than untreated seedlings. Treatment with 2,4-D did not change the RQ which remained near 1.0. These results suggested that carbohydrate was the major substrate being oxidized in both treated and control seedlings. Subsequent work, as yet unpublished, has shown that 2,4-D treated seedlings did not contain more carbohydrate (reducing sugars, sucrose, and starch) than the untreated seedlings. 2,4-D, then, affects the respiratory rate of pea seedlings without changing the RQ or increasing the carbohydrate level. It would be of interest, therefore, to ascertain whether or not 2,4-D causes glucose to be catabolized via a different pathway, or, if a number of pathways are operative, to cause a change in the amount of glucose catabolized via one of the various pathways.

Recently Beevers and Gibbs (2) have shown that the pentose phosphate pathway (oxidative pathway or hexose monophosphate shunt) may be a major route of glucose catabolism in a number of higher plants including peas. On the other hand, these workers obtained evidence by two independent methods (2, 3) that in corn root tips the Embden-Meyerhof-Parnas (E.P.M.) glycolytic pathway is the only operative one. Beevers and Gibbs applied a procedure developed by Bloom and Stetten (4) in which the initial yields of $C^{14}O_2$ from tissues supplied with glucose-1-$C^{14}$ (G-1-$C^{14}$) and glucose-6-$C^{14}$ (G-6-$C^{14}$) are compared. If glucose were broken down via the E.M.P. pathway, the rate of $C^{14}O_2$ production from the first and the sixth carbon of the glucose molecule should be the same. If, on the other hand, glucose were broken down via the pentose phosphate pathway the rate of $C^{14}O_2$ production from the first carbon of the glucose molecule would initially be greater than that from the sixth carbon. The use of the ratio

$$\frac{\text{fraction of } C^{14} \text{ added as G-6-}C^{14} \text{ appearing as } C^{14}O_2}{\text{fraction of } C^{14} \text{ added as G-1-}C^{14} \text{ appearing as } C^{14}O_2}$$

($C_6/C_1$ ratio) to give directly the maximal fraction of glucose catabolized via the E.M.P. pathway has been suggested by Bloom and Stetten (4). This assumes that all the $C^{14}O_2$ obtained from G-6-$C^{14}$ is a result of the working of the E.M.P. pathway. If the pentose phosphate pathway also contributes to the yield of $C^{14}O_2$ from G-6-$C^{14}$, then the fraction of glucose catabolized via the E.M.P. pathway is less than the $C_6/C_1$ ratio. For a more complete discussion of this method see Bloom, Stetten and Stetten (6), Bloom and Stetten (4, 5) and Beevers and Gibbs (2).

This paper will report the results of a study on the effect of 2,4-D on the participation of the pentose phosphate pathway in glucose catabolism of root tips from corn and pea seedlings using the G-6-$C^{14}$/G-1-$C^{14}$ method of Bloom and Stetten. The action of 2,4-D is compared to that of indoleacetic acid (IAA).

## MATERIALS AND METHODS

Dry pea seeds (*Pisum sativum* L., var. Emerald) were soaked overnight in distilled water with aeration, planted in moist sand and placed in the dark at 20° C for approximately 60 hours. During this time the seedlings reached a length of 4 to 6 cm, most of which was unbranched primary root. The seedlings were removed from the sand, washed in distilled water and placed in vials containing either $10^{-2}$ M potassium phosphate buffer, pH 5.3 or buffer plus $10^{-3}$ M 2,4-D. Only the distal 1 to 2 cm of the root were immersed in the solution. The vials were placed in the dark at 20 to 21° C for 12 hours after which period the seedlings were removed from the vials, washed in distilled water, blotted on filter paper, and approximately 2 cm of the root tip cut off. The root tips were weighed in groups of seven. The length of the individual root tips within each group was adjusted so that the weights of the groups within each experiment were the same within two mg.

Corn seeds (*Zea Mays* L., var. Funks G-50) were soaked overnight in distilled water with aeration and then placed in Pyrex trays on moist filter paper. The trays, covered with a sheet of glass, were placed in the dark at 20 to 21° C for approximately 60 hours. The corn seedlings were handled and treated in the same manner as the pea seedlings.

Oats (*Avena sativum* L., var. Seminole) were germinated in sand as were the peas. Since the length and fragility of the many roots made them difficult to handle, only one experiment was run using oat root tips.

Pea and corn seedlings were treated with buffered 2,4-D or buffer alone, both with and without the food storage organs (cotyledons or endosperm) attached. The corn endosperm and scutellum were removed with a razor; the pea cotyledons were snapped off.

In all experiments eight equal samples of root tips (each sample weighed about 0.2 gm) were placed in standard 15-ml conical Warburg vessels. The vessels contained 2 ml of $10^{-2}$ M potassium phosphate buffer, pH 5.3 (main compartment), 0.2 ml of 20% KOH (center well), and 0.2 ml of $10\,N$ $H_2SO_4$ (side arm). Four vessels contained root tips from 2,4-D treated

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seedlings and four vessels contained root tips from buffer treated seedlings. Two of the four vessels in each group received 0.1 ml of G-1-$C^{14}$; the other two vessels in each group received 0.1 ml of G-6-$C^{14}$. The added radioactive glucose had an activity of approximately 20,000 cpm. One-tenth ml of radioactive glucose solution contained 0.8 mg of glucose. Immediately after adding the glucose the vessels were attached to the manometers and placed in the constant temperature bath. Air was the gas phase in the vessels. The vessels were shaken in the dark at 25° C for three and one half hours. At the end of this period the acid was tipped from the side arm into the main compartment to stop the reactions and release bound $CO_2$. Shaking was continued for 20 minutes, after which the contents of the center well were removed with washing, and the respiratory $CO_2$ was precipitated as $BaCO_3$ with 25 % barium acetate. The precipitate was washed two times with 15-ml portions of 50 % ethanol, then plated on stainless steel planchets and counted with a windowless gas-flow counter.

In the experiments in which seedlings were treated with IAA, all operations were the same as in the experiments with 2,4-D.

2,4-D (Eastman Kodak Co.) was neutralized with NaOH, and the sodium salt recrystallized two times from a water-alcohol solution. IAA (reagent grade) was purchased from Fisher Scientific Company, and was used without further purification. G-1-$C^{14}$ and G-6-$C^{14}$ were purchased from Volk Radiochemical Company.

## Results and Discussion

2,4-D Treatment: In table I the percent of added $C^{14}$ recovered as $C^{14}O_2$ from G-1-$C^{14}$ and G-6-$C^{14}$, and the $C_6/C_1$ ratios derived from these yields are compared for root tips from buffer treated and 2,4-D treated seedlings. In all cases the $C_6/C_1$ ratio was markedly lowered as a result of 2,4-D treatment. A lower ratio could result from a lower yield of $C^{14}O_2$ from G-6-$C^{14}$ (the yield from G-1-$C^{14}$ remaining unchanged), from a higher yield of $C^{14}O_2$ from G-1-$C^{14}$ (the yield from G-6-$C^{14}$ remaining unchanged), or

Table I

Effect of 2,4-D Pre-Treatment of Pea, Corn and Oat Seedlings on the Metabolism of Radioactive Glucose

| Expt | Tissue * | Treatment † | % $C^{14}$ added recovered as $C^{14}O_2$ | | $C_6/C_1$ |
|---|---|---|---|---|---|
| | | | Glucose-1-$C^{14}$ | Glucose-6-$C^{14}$ | |
| 1 ** | Pea root tips (minus cotyledons) | Buffer | 17.3 | 7.4 | 0.43 |
| | | 2,4-D + Buffer | 30.9 | 8.7 | 0.28 |
| 2 | " | Buffer | 6.8 | 4.0 | 0.59 |
| | | 2,4-D + Buffer | 11.7 | 2.7 | 0.23 |
| 3 | " | Buffer | 5.5 | 4.4 | 0.80 |
| | | 2,4-D + Buffer | 12.3 | 5.6 | 0.46 |
| 4 | Pea root tip (intact) | Buffer | 11.9 | 6.2 | 0.53 |
| | | 2,4-D + Buffer | 12.7 | 1.4 | 0.11 |
| 5 | " | Buffer | 6.2 | 4.3 | 0.70 |
| | | 2,4-D + Buffer | 9.6 | 3.0 | 0.31 |
| 6 | " | Buffer | 4.9 | 4.3 | 0.88 |
| | | 2,4-D + Buffer | 5.1 | 1.9 | 0.37 |
| 7 | " | Buffer | 5.4 | 4.6 | 0.85 |
| | | 2,4-D + Buffer | 9.6 | 2.9 | 0.30 |
| 8 | Corn root tip (intact) | Buffer | 7.1 | 5.2 | 0.73 |
| | | 2,4-D + Buffer | 14.5 | 4.1 | 0.29 |
| 9 | " | Buffer | 10.4 | 5.5 | 0.53 |
| | | 2,4-D + Buffer | 26.5 | 6.3 | 0.24 |
| 10 | " | Buffer | 11.6 | 5.5 | 0.47 |
| | | 2,4-D + Buffer | 24.3 | 5.9 | 0.24 |
| 11 | Corn root tip (minus endosperm) | Buffer | 4.8 | 4.9 | 1.02 |
| | | 2,4-D + Buffer | 10.7 | 4.7 | 0.44 |
| 12 | " | Buffer | 7.3 | 4.5 | 0.62 |
| | | 2,4-D + Buffer | 20.0 | 6.6 | 0.33 |
| 13 | Oat root tip (intact) | Buffer | 5.2 | 4.3 | 0.83 |
| | | 2,4-D + Buffer | 14.8 | 4.6 | 0.31 |

* Words in parentheses refer to whether or not seedlings were treated with cotyledons (or endosperm) attached or removed.

** In this experiment the vessels were shaken for 4 hours (instead of 3.5 hrs) before tipping in the acid.

† Seedlings placed in vials in the dark so that the distal 1 to 2 cm of the root were covered with phosphate buffer ($10^{-2}$ M, pH 5.3) or with 2,4-D ($10^{-3}$ M) plus buffer ($10^{-2}$ M, pH 5.3). Treatment was for 12 hours at 20° C.

from the combination of a higher yield with G-1-$C^{14}$ and a lower yield from G-6-$C^{14}$. The results given in table I illustrate all three situations.

The results obtained with pea root tips varied according to the way in which the seedlings were treated. Thus, root tips obtained from intact seedlings behaved differently than those obtained from seedlings which, prior to 2,4-D treatment, had their cotyledons removed. In the first three experiments (cotyledons removed before treatment) the lower ratio was primarily a result of an increase in the yield of $C^{14}O_2$ from G-1-$C^{14}$. The yield from G-6-$C^{14}$ was not greatly affected by 2,4-D treatment except in experiment 2, where a 32% decrease was obtained. Even in this instance, however, the increase in the yield of $C^{14}O_2$ from G-1-$C^{14}$ (72%) was the greater change. In experiments 4 through 7 (cotyledons attached during treatment) the lower ratio resulted either from a decrease in the yield of $C^{14}O_2$ from G-6-$C^{14}$ (experiments 4 and 6) or from both a decrease in the yield from G-6-$C^{14}$ and an increase in the yield from G-1-$C^{14}$ (experiments 5 and 7).

These results suggest that in root tips of pea seedlings the action of 2,4-D on the metabolism of glucose is controlled in some manner by the level of food reserves in the seedling during the treatment period. At high levels of food reserves (cotyledons attached) 2,4-D causes a decrease in the yield of $C^{14}O_2$ from G-6-$C^{14}$. At low levels of food reserves (cotyledons removed) 2,4-D causes an increase in the yield of $C^{14}O_2$ from G-1-$C^{14}$. The results of experiments 2, 5 and 7 represent perhaps a transition stage in which 2,4-D causes both an increase in the yield from G-1-$C^{14}$ and a decrease in the yield from G-6-$C^{14}$. It is realized, of course, that the cotyledons may supply the root with substances, not foodstuffs, which modify the action of 2,4-D.

In contrast to peas the action of 2,4-D on monocots (corn and oats) was not apparently influenced by the level of the food reserves. Thus with corn, 2,4-D treatment caused an increase (more than 100 percent) in the yield of $C^{14}O_2$ from G-1-$C^{14}$, but did not appreciably affect the yield from G-6-$C^{14}$. This was true regardless of whether or not the endosperm was left on during 2,4-D treatment of the seedlings.

The increased production of $C^{14}O_2$ from G-1-$C^{14}$ in root tips of corn and oat seedlings and in the root tips of pea seedlings (cotyledons removed) as a result of 2,4-D treatment suggests that the action of 2,4-D is to cause an increase in the amount of glucose catabolized via the pentose phosphate pathway; the amount of glucose catabolized via the E.M.P. pathway remaining unchanged. In contrast, the results of experiments 4 through 7 (table I, pea seedlings treated with cotyledons attached) suggest that 2,4-D causes a decrease in the amount of glucose catabolized via the E.M.P. pathway while, at the same time, increasing the amount catabolized via the pentose phosphate pathway. One of the assumptions necessary in assessing the degree of participation of the E.M.P. and pentose phosphate pathways from the $C_6/C_1$ ratio is that all $C^{14}O_2$ obtained from G-6-$C^{14}$ results from glucose catabolized via the E.M.P. pathway. Thus it is assumed that a glucose molecule catabolized via the pentose phosphate pathway, although it would lose its first carbon rapidly, would lose its sixth carbon slowly—so slowly, in fact, that in short time experiments essentially none would be lost. The results shown in table I (particularly those obtained using root tips from corn and oat seedlings) substantiate this assumption. In these experiments 2,4-D had very little effect on the yield of $C^{14}O_2$ from G-6-$C^{14}$ while greatly increasing the yield from G-1-$C^{14}$. If the extra $C^{14}O_2$ liberated from G-1-$C^{14}$ indicates that more glucose was being catabolized via the pentose phosphate pathway, then, if the above assumption did not hold true, more $C^{14}O_2$ should also have been liberated from G-6-$C^{14}$. This was not the case. Small increases in the yield of $C^{14}O_2$ from G-6-$C^{14}$ were obtained in experiments 1, 3 and 12 which indicate that the experimental time period (3.5 hrs) is close to the maximum allowable if significant $C_6/C_1$ ratios are to be obtained. Of course, if 2,4-D inhibited the E.M.P. pathway, the yield of $C^{14}O_2$ from G-6-$C^{14}$ resulting from the contributions of both pathways could conceivably equal the yield obtained with the untreated root tips. Such a fortuitous circumstance is unlikely.

Such interpretations are attractive, but, due to the many transformations and interconversions of carbohydrates which are known to occur in plants, are presented here only as tentative ones. Indeed, some of these results would also be obtained if only the initial reactions of the pentose phosphate pathway leading to the decarboxylation of 6-phosphogluconate (and not the operation of the entire pathway) were increased by the action of 2,4-D. Axelrod and Beevers (1) have recently reviewed the literature on the occurrence of the pentose phosphate pathway in plants. They discuss in detail the difficulty of evaluating the degree of participation of the pathway in respiration from the $C_6/C_1$ ratio.

Beevers and Gibbs (2, 3) have concluded from results obtained by two independent methods that in corn root tips glucose is catabolized almost entirely via the classical E.M.P. pathway. The results presented in this paper do not wholly support such a conclusion. The $C_6/C_1$ values (table I) obtained with root tips from buffer treated corn seedlings varied over a wide range (0.47 to 1.02). This variation seemed to be correlated with the moisture conditions under which the seedlings were grown. If the corn was germinated under moist to dry conditions the primary roots were thickly covered with root hairs; $C_6/C_1$ ratios obtained from root tips of these seedlings were low (0.5 to 0.6). If the corn was grown under moist to wet conditions (under wet conditions there was a film of free water on the surface of the filter paper on which the seeds were germinated) the primary roots had very few root hairs; $C_6/C_1$ ratios obtained with root tips from these seedlings were high (0.7 to 1.0). Assuming the $C_6/C_1$ ratio to be a true measure of the fraction of glucose respired by the

TABLE II

EFFECT OF IAA PRE-TREATMENT OF PEA AND CORN SEEDLINGS ON THE METABOLISM OF RADIOACTIVE GLUCOSE

| EXPT | TISSUE * | TREATMENT † | % $C^{14}$ ADDED RECOVERED AS $C^{14}O_2$ | | $C_6/C_1$ |
|---|---|---|---|---|---|
| | | | GLUCOSE-1-$C^{14}$ | GLUCOSE-6-$C^{14}$ | |
| 1 | Pea root tips (minus cotyledons) | Buffer | 8.0 | 5.0 | 0.63 |
| | | IAA + Buffer | 7.8 | 4.8 | 0.62 |
| 2 | " | Buffer | 7.9 | 4.6 | 0.58 |
| | | IAA + Buffer | 8.5 | 3.9 | 0.46 |
| 3 ** | " | Buffer | 5.7 | 4.9 | 0.86 |
| | | IAA + Buffer | 5.9 | 5.1 | 0.86 |
| 4 | Pea root tips (intact) | Buffer | 8.7 | 5.6 | 0.64 |
| | | IAA + Buffer | 8.0 | 3.3 | 0.41 |
| 5 | " | Buffer | 7.3 | 5.3 | 0.73 |
| | | IAA + Buffer | 5.1 | 2.8 | 0.55 |
| 6 | " | Buffer | 8.0 | 4.9 | 0.61 |
| | | IAA + Buffer | 7.7 | 3.4 | 0.44 |
| 7 | Corn root tips (intact) | Buffer | 6.4 | 5.5 | 0.86 |
| | | IAA + Buffer | 6.3 | 2.1 | 0.40 |

* Words in parentheses refer to whether or not the cotyledons (or endosperm) were removed before treatment.
** In this experiment $10^{-10}$ M IAA was used.
† Seedlings placed in vials in the dark so that the distal 1 to 2 cm of the root were covered with phosphate buffer ($10^{-2}$ M, pH 5.3) or with IAA ($10^{-5}$ M) plus buffer ($10^{-2}$ M, pH 5.3). Treatment was for 12 hours at 20° C.

E.M.P. pathway then, only if corn is germinated under conditions of high moisture, does the E.M.P. pathway become the dominant one in glucose catabolism. A wide range of $C_6/C_1$ ratios (0.43 to 0.88) was also obtained with root tips from buffer treated pea seedlings, but, since these seedlings were germinated in sand, no such correlation between moisture conditions and magnitude of the $C_6/C_1$ ratio was made.

It should be noted, in this connection, that the variation in the $C_6/C_1$ ratios obtained from buffer treated root tips of both peas and corn indicate only a variation in the contributions of the two pathways to the total glucose oxidation. They do not indicate variations in the rate of glucose oxidation. Although the data on the yields of $C^{14}O_2$ give an indication of the rate of glucose oxidation, it is difficult, because of variations in the weight of tissue used and because of variations in the amount of endogenous glucose, to compare the yields of $C^{14}O_2$ between experiments. Even though all root tips were approximately 2 cm in length they varied in thickness from one batch of seedlings to the next and, as a result, there was a variation of as much as 25 % in weight of root tips between experiments. The amount of endogenous glucose in root tips of different batches of seedlings also varied; which variation, in the case of pea root tips, was as high as 20 %. A comparison of the data within a single experiment can be made, however, and it shows clearly that, except for root tips from pea seedlings treated with the cotyledons attached, 2,4-D caused an increase in the amount of glucose oxidized and that this extra glucose was oxidized via the pentose phosphate pathway.

IAA TREATMENT: The effects of IAA pre-treatment of pea and corn seedlings on the $C_6/C_1$ ratio are shown in table II. The results obtained with IAA differ from those obtained with 2,4-D in three ways: 1) In no case did IAA cause a significant increase in the yield of $C^{14}O_2$ from G-1-$C^{14}$. 2) In the experiments with pea seedlings from which the cotyledons were removed before treatment, IAA had essentially no effect on the yields of $C^{14}O_2$ from either G-1-$C^{14}$ or G-6-$C^{14}$. This was true both when high concentrations ($10^{-3}$ M) and when low concentrations ($10^{-10}$ M) of IAA were used. Root growth is strongly inhibited by an IAA concentration of $10^{-3}$ M, but a maximal rate of elongation is obtained at an IAA concentration of $10^{-10}$ M (9). 3) With corn root tips the yield of $C^{14}O_2$ from G-6-$C^{14}$ was greatly decreased as a result of IAA treatment. 2,4-D, on the other hand, had no effect on the yield of $C^{14}O_2$ from G-6-$C^{14}$ in corn root tips.

One point of similarity is apparent from the results shown in table II. When pea seedlings treated with the cotyledons attached were used, the action of IAA was much the same as was that of 2,4-D.

From these results it is concluded that IAA does not influence glucose catabolism in the same manner as does 2,4-D.

Gibbs and Beevers (7), using oat coleoptiles, found that although IAA increased the consumption of glucose it did not change the $C_6/C_1$ ratio.

## SUMMARY

The yields of $C^{14}O_2$ from glucose-1-$C^{14}$ and glucose-6-$C^{14}$ in short time experiments were used to evaluate the participation of the Embden-Meyerhof-

Parnas (glycolytic) and the pentose phosphate pathways of glucose catabolism in root tips of 2,4-D treated pea, corn and oat seedlings.

From the results obtained the following tentative conclusions were drawn.

1. In all seedlings tested 2,4-D caused an increase in the amount of glucose catabolized via the pentose phosphate pathway.

2. In corn and oat seedlings and in pea seedlings which had their cotyledons removed prior to 2,4-D treatment, the amount of glucose catabolized via the classical glycolytic pathway was not affected by 2,4-D treatment. In contrast, 2,4-D caused a decrease in the amount of glucose catabolized via the glycolytic pathway in pea seedlings which were 2,4-D treated with the cotyledons attached.

3. The fraction of glucose catabolized via the Embden-Meyerhof-Parnas pathway in root tips of untreated corn seedlings varied with the moisture conditions under which the seeds were germinated.

The effect of IAA on pathways of glucose catabolism was also studied. It was concluded that IAA does not influence glucose catabolism in the same manner as does 2,4-D.

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## BRIEF PAPERS

# COMPARATIVE ABSORPTION OF COBALT-60 BY UPPER AND LOWER EPIDERMIS OF LEAVES[1,2,3]

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There is considerable controversy as to whether chemicals applied to leaves enter mainly through the stomata. In a recent review Boynton (1) concluded his discussion by the statement: "It may be tentatively concluded that aqueous solutions both penetrate the leaf through the cuticle and make initial entry into the leaf by way of the stomates. While the latter may be of greatest significance in short periods of time, the former may be as effective a means of entrance over longer times." More recently Volk and McAuliffe (7) and Teubner et al (5) have concluded that stomatal openings are not the major sites of entry into the leaf. On the other hand Gustafson (2), Gustafson and Schlessinger (3) and Skoss (4) present evidence that stomata may play an important part in the entrance of solutes from aqueous solutions.

In this investigation the following plants were used: *Tropaeolum majus*, *Phaseolus vulgaris*, var. Black Valentine, *Gossypium* sp., var. Empire, *Cucumis sativus*, var. Early Fortune, *Brassica oleracea*, var. Early Jersey, *Glycine Max*, var. Biloxi, and *Clivia miniata*. The plants were greenhouse grown and carefully selected and paired. One of a pair had the solution applied to the lower surface and the other to the upper surface.

Stomatal counts were made on both upper and lower surfaces of 5 species. The stomatal count was made by applying a thin coat of Duco cement to the surface of the leaf. In a few minutes the cement dried and could be peeled off, mounted dry under a cover glass and held in place by cement at the corners of the glass. If the cement was properly applied the stomata stood out very clearly and could be counted easily, under an ordinary microscope.

Cobalt-60, as the chloride, was used as the test element to determine the absorption and translocation within the plant. Throughout the investigation, solutions having an activity of 30.2 or 30.7 $\mu$c/ml were used, and 0.05 ml of this solution was applied per plant in 2 drops, which gave a quantity of active cobalt equivalent to 0.22 $\mu$gm per plant. The drops were placed on either side of the main vein near the petiole. Tween-20 was incorporated into the solution as a spreader, in a concentration of 0.05 %. The pH of this solution was 4. The Tween-20 left a residue which was readily visible. The absorption period was always 6 hours, and usually from 10:00 A.M. to 4:00 P.M. The leaf to which the solution was applied varied considerably in the different experiments, but the leaf chosen was always full grown, healthy and in the same position within an experiment. The leaf used depended frequently upon the age of the plant, thus in the three experiments with cotton a cotyledon, the first and the third true leaf were used. One of the primary leaves was always employed in the bean.

At the time of harvest a disk of leaf tissue was removed at the location of each drop. This disk was larger than the drop by several millimeters on all sides. These disks were discarded. The plants were then divided into several portions, to be dried and counted separately. The material was dried at 90° C for about 24 hours, ground and stored until counted.

[1] Received August 31, 1956.

[2] Paper No. 1064, Department of Botany, University of Michigan, Ann Arbor, Michigan.

[3] This investigation has been aided financially by the Michigan Memorial-Phoenix Project of the University of Michigan.

TABLE I

COMPARATIVE ABSORPTION AND TRANSLOCATION OF $Co^{60}$ WHEN APPLIED TO THE UPPER OR LOWER SURFACE OF THE LEAF

| PLANT | NO. OF PAIRS OF PLANTS USED | NO. OF EXPTS. | MEAN CPM/PLANT WHEN Co APPLIED | | $t$* |
|---|---|---|---|---|---|
| | | | UPPER SURFACE | LOWER SURFACE | |
| *Tropaeolum majus* | 21 | 5 | 420 | 431 | −0.20 |
| *Phaseolus vulgaris*, var. Black Valentine | 31 | 6 | 511 | 638 | −2.05** |
| *Gossypium* sp., var. Empire | 9 | 3 | 142 | 130 | +0.30 |
| *Cucumis sativus*, var. Early Fortune | 18 | 4 | 299 | 309 | −0.23 |
| *Brassica oleracea*, var. Early Jersey | 9 | 2 | 1217 | 997 | +1.25 |
| *Glycine Max*, var. Biloxi | 14 | 3 | 414 | 623 | −5.39*** |
| *Clivia miniata* | 3 | 3 | 202 | 205 | −0.03 |

* The value $t$ is that obtained by the Student-Fisher procedure applied to the averages of differences obtained by taking the total counts for a plant when the cobalt was applied to the upper surface of leaves minus the total counts for a matched plant when the cobalt was applied to the lower surface of the leaf.

** Significant at 5 % level.

*** Significant at 0.1 % level.

The gamma-ray emission from the cobalt-60 was determined with a scintillation counter. Preliminary experiments showed there was no self absorption.

The difference of the means of absorption by the upper and the lower surfaces has been evaluated by the Student-Fisher *t*-test and are recorded in table I. An examination of the table shows that in two instances the difference in absorption is significantly greater by the lower surface; in the other four species there is no significant difference. Special attention should be called to *Clivia miniata*, which has no stomata on the adaxial (upper) surface, yet the absorption is as great as that by the abaxial surface. The stomatal counts are given in table II, and it is evident that in all instances the lower surfaces have many more stomata than the upper surfaces.

TABLE II

NUMBER OF STOMATA PER SQUARE CENTIMETER IN THE UPPER AND LOWER EPIDERMIS

| PLANT | STOMATA/CM² | |
|---|---|---|
| | UPPER EPIDERMIS | LOWER EPIDERMIS |
| *Tropaeolum majus* | 2,000 | 12,140 |
| *Phaseolus vulgaris*, var. Black Valentine | 4,413 | 24,960 |
| *Brassica oleracea*, var. Early Jersey | 21,668 | 33,494 |
| *Glycine Max*, var. Biloxi | 5,397 | 19,207 |
| *Clivia miniata* | None | 3,654 |

The data presented in tables I and II do not support any hypothesis that the entrance of solute into the leaf is predominantly through the stomata but do not preclude the possibility that in some plants stomata may play a part. In the case of soybean and Black Valentine bean there was significantly more cobalt absorbed by the lower surface than by the upper surface. In the other plants there was no significant difference in the absorption by the two surfaces.

## SUMMARY

From the conflicting evidence one finds in the literature as well as that presented here, this author is coming to believe that there is a difference in plants, as to the rate of absorption by the two surfaces of the leaf. Some absorb more rapidly through the lower surface like the soybean, others more rapidly through the upper surface as does the cabbage. This difference need not be associated with stomata and in some instances as in *Clivia miniata* there is certainly no connection between absorption and stomata. The cuticle is generally considered to be thicker on the upper surface than on the lower surface and this differential would be a factor in rate of absorption. In a recent review Van Overbeek (6) presents the theories on penetration through the cuticle.

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# AN ELECTRONIC TEMPERATURE INTEGRATOR FOR PLANT PHYSIOLOGICAL RESEARCH

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The great need for complete climatological records in plant physiological research has been re-emphasized during the present period of extensive research on the use of herbicides. The temperature factor is of special interest in herbicide investigations because of its dual effects on plant response and rate of breakdown of the chemicals in the soil and on the surfaces of plants. The experimental electronic temperature integrator described herein was assembled in an effort to provide a convenient and adaptable unit to meet the needs of such research. The instrument has proved useful in the summer temperature range.

The method formulated by Appleman and Eaton (1) for expressing the temperature factor in degree-hours in agricultural research has proved useful in connection with herbicide research at this station. These cumulative temperature records from any one experiment may be compared readily with that of any other experiment. They may be prepared to represent the total temperature effect experienced during

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an investigation or may represent any time interval desired to coincide with crop growth phases or the activity periods of the various herbicides being studied. The electronic temperature integrator facilitates the preparation of these cumulative temperature records.

The basic circuit of the temperature integrator shown in figure 1 A was derived from the sunlight integrator circuit of Sprague and Williams (3, 4) by substituting a thermistor for the phototube as a current regulating mechanism. The original sunlight integrator circuit of Sprague and Williams was modified by the Weather Instruments Committee of the Southeastern Regional Soils-Weather Project (Regional R-M Project S-5) and certain of these modifications are used in the temperature integrator circuit. An evaluation of the performance and general research utility of sunlight integrators by Somers and Hamner (2) has shown that an acceptable level of reliability can be obtained with electronic equipment of this general type.

The organization of the equipment into a compact unit is shown in figure 2 which also shows the receptor tube containing the thermistor and the connecting co-axial cable. Details of the receptor tube construction are shown in figure 3 A. The length of the glass or plastic tube may be varied to allow placement of the receptor tip at any desired depth in the soil. When the unit was used to measure air temperatures, the thermistor was shaded from direct or reflected sunlight to avoid the heating effects of radiant energy of the sun on the thermistor bead, as this caused erroneous results. This shading was obtained with a supplementary aluminum foil sleeve which extended beyond the tip of the receptor and was of a large enough diameter to allow free air flow through the open end. Air in the receptor tube was kept free of moisture by the use of silica gel. The indicator type of gel which changes color, blue to pink, as the air in the tube becomes moist, was used. Accumulation of moisture in the tube may be discovered at a glance and the drying agent changed.

The relay tube $T_1$, the condenser $C_1$, and the resistor $R_2$ were mounted in a gasket-sealed dry chamber containing silica gel drying agent. This precaution was necessary to prevent electrical leakage due to the accumulation of a surface moisture film on these parts.

The count interval at 80° F was adjusted to bring it within the range of 10 to 15 seconds between counts. This was accomplished by changing the value of resistor $R_2$ until the desired count interval was achieved at this temperature. The resistor value required will usually be from 5 to 10 megohms but must be determined by trial. Adjustment of the count interval to these recommended values allows any 24-hour record to be accumulated within the limits of one complete revolution of the 4-digit Western Electric Message Register indicated.

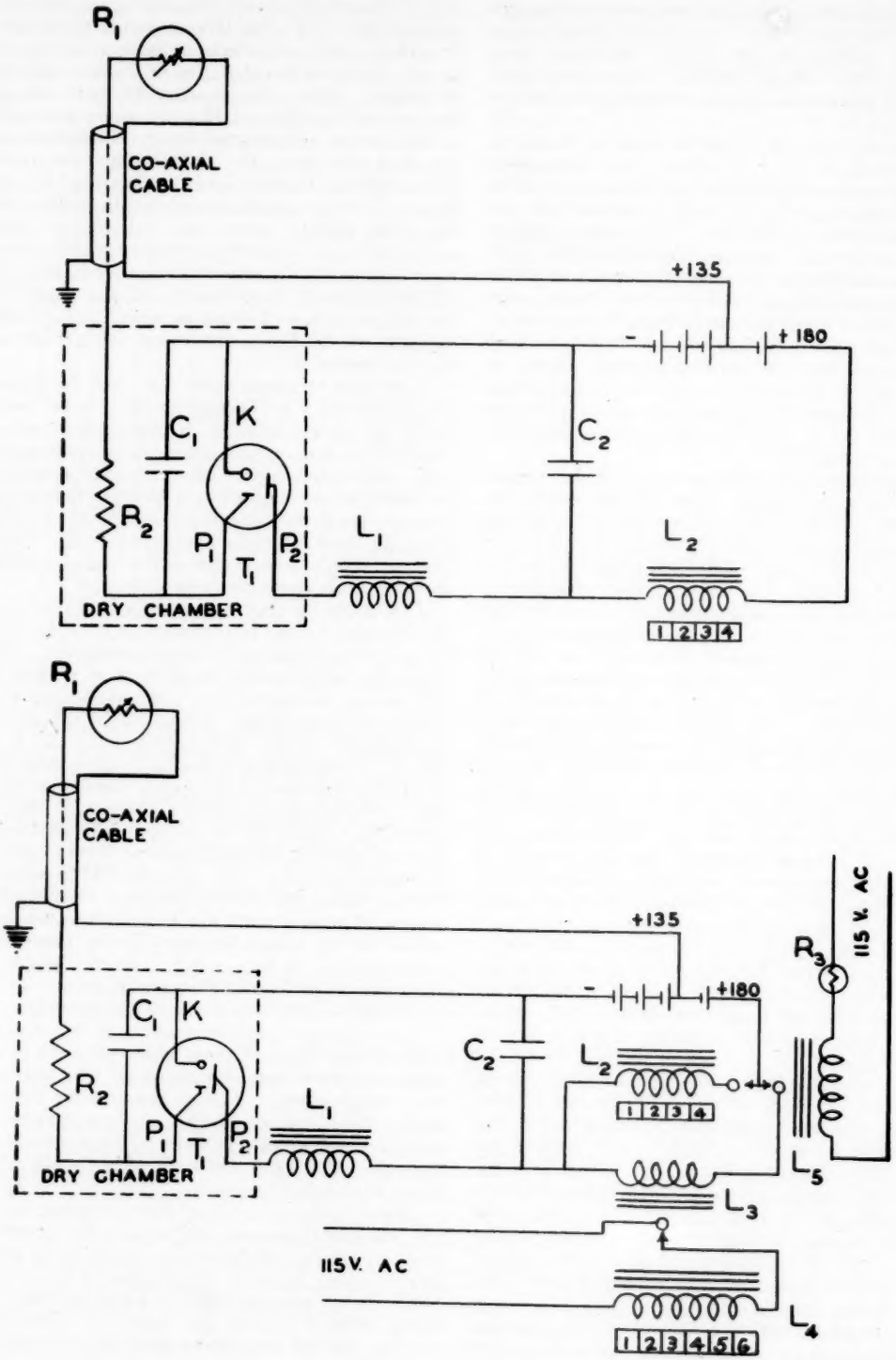
Units placed where a 115-volt AC supply was available made use of the additional convenience of a 6-digit Wizard AC counter by utilizing two relays as shown in figure 1 B. The Wizard counter can be reset to zero and will accumulate as much as a 3-month summer record within the limits of a single complete revolution. When the Western Electric Message Register and the 115-volt Wizard counter were used in combination, as shown, the Western Electric counter was read each time the Wizard counter was read. The difference between successive readings on the Western Electric counter represented the periods during which the AC service was interrupted. This method of using the battery operated counter through an automatic relay made certain that a complete record was obtained. Removing the AC line plug made the unit portable as the counting circuit shifted automatically to the DC counter when the AC current was interrupted.

Instrument readings were converted to degree-hours above 0° F by multiplying by a factor determined for each instrument by comparison with a Bendix-Friez thermograph record obtained simultaneously. The most accurate determinations of this factor were obtained when wide temperature fluctuations occurred during the calibration period of 2 to 3 weeks. When the count interval was adjusted to 10 to 15 seconds at 80° F, the factor for conversion of readings to degree hours was in the range of 0.2 to 0.3.

Laboratory calibration data representative of the performance of these instruments are given in figure 3 B where the results of a linearity study of a single instrument are presented in the form of a graph of time interval between counts in seconds against temperature. An acceptable straight line relationship is shown in the range from approximately 65° F to 115° F. Comparison of a Bendix-Friez thermograph record and the above unit within this temperature range over a period of 56 days, where daily calculations were made, gave an average daily conversion factor of $0.2150 \pm 0.0046$. The count interval for this instrument at 80° F was approximately 5 seconds. This instrument was later adjusted to a count interval of 10 to 15 seconds at 80° F in order to meet the limitations of the 4-digit Western Electric counter in accumulating a 24-hour record. Linearity studies at this longer count interval gave similar results.

Further studies were conducted to determine the variations among integrator units. This was done by constructing a total of three additional units in the manner described here and comparing their performances simultaneously with a Bendix-Friez thermograph. The close correlation of these instruments with the thermograph was shown by correlation coefficients of 0.944, 0.918, and 0.964 where $n = 20$. The values were highly significant.

Observations on an instrument operating over a two year period demonstrated the need for a monthly check on battery voltage levels. It was found necessary to replace batteries which registered less than 40 volts. A complete change of batteries every six months insured trouble free operation. The relay tube was changed annually to avoid difficulties due to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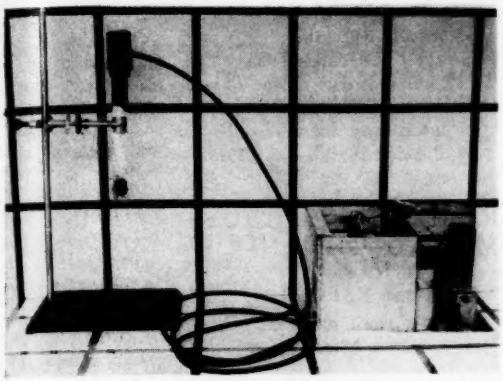

FIG. 2. The electronic temperature integrator unit is shown with cover removed. The cover slips over the unit and is held in place by metal screws. A small plastic window above the counter allows convenient observation. The co-axial cable and receptor unit are also shown. Background is marked off in 6-inch squares to indicate actual and relative size of the various parts of the unit.

tube failure, though experience with this tube in sunlight integrators indicates a much longer life than one year.

## SUMMARY

The circuit, construction data, and performance of an experimental electronic temperature integrator are presented. This circuit is based on the electronic sunlight integrator of Sprague and Williams (3, 4) and substitutes a thermistor for the photo tube used in the sunlight integrator. The instruments are portable and convenient for field or greenhouse use. A straight line response is obtained in the range from 65 to 115° F (ca 18 to 46° C). The portable units must be read daily but an alternate circuit using an AC counter may be read at weekly or monthly intervals. High correlation values were obtained in this temperature range between three instruments and a Bendix-Friez thermograph ($r = 0.944$, $r = 0.918$, $r = 0.964$, $n = 20$). The instruments are especially useful in preparing degree-hour records in plant physiological research.

Acknowledgement is made by the authors to P. R. Chambers of the Virginia Truck Experiment Station for assistance in the construction and calibration of these instruments.

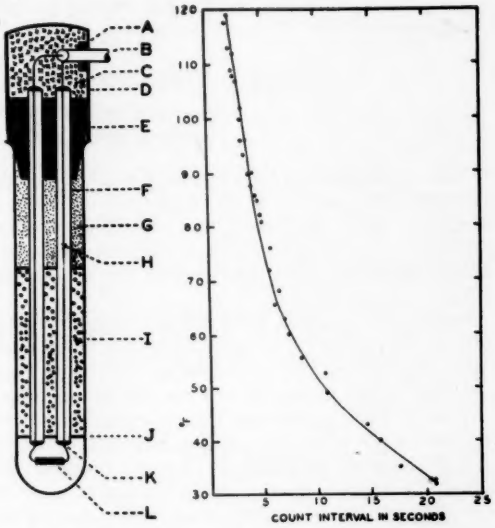


FIG. 3 A (*left*). The electronic temperature integrator receptor unit construction. A—Tape to hold co-axial cable in place. B—Co-axial cable. C—Waterproof packing (Johns-Mansville Duxseal). D—Plastic water pipe section fitted snugly around rubber stopper. E—Rubber stopper. F—Capillary glass tube. G—Silica gel drying agent. H—Fine copper wire. I—Glass wool. J—Rubber washer. K—Sealing wax. L—Thermistor.

FIG. 3 B (*right*). Calibration curve of an electronic temperature integrator unit where count interval in seconds is graphed against temperature on the Fahrenheit scale.

FIG. 1 A (*upper diagram*). Circuit of the completely battery powered portable electronic temperature integrator. Parts list:

$R_1$—VECO 71A2 Thermistor (Victory Engineering Corp., Springfield Road, Union, N. J.).

$R_2$—Carbon resistor, 1 watt, 5 to 10 megohms to adjust count interval to 10 to 15 sec at 80° F.

$C_1$—Condenser, 1 mfd., 400 V (highest quality obtainable).

$C_2$—Condenser, 4 mfd., 400 V.

$L_1$—Choke coil, 5 to 12 henries, 100 to 400 ohms, lower resistance preferable.

$L_2$—Electric counter, Western Electric Message Register, Model 5AA or 14LB (Graybar Electric Co.).

$T_1$—RCA OA4G or equivalent, with base removed, Western Electric 313C Relay Tube may also be used.

Batteries, four (45 V) B batteries, Eveready No. 482 or equivalent.

FIG. 1 B (*lower diagram*). Circuit of the electronic temperature integrator with alternate circuit employing a 115 V AC counter. Parts list for AC counter circuit:

$L_3$—Potter and Bromfield Relay LM 11 DPDT 10,000 ohms.

$L_4$—Wizard Panelmount Electric Counter PEC-9, 115 V, 60 ~, AC. (Production Instrument Co., Chicago, Ill.).

$L_5$—Potter and Bromfield Relay PR 11 A DPDT. 115 V, 50 to 60 ~, AC.

$R_3$—Incandescent lamp, 7.5 watt (to reduce voltage to relay coil and thus reduce heating).

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# STUDIES OF MALEIC HYDRAZIDE INTERACTIONS WITH THIOL COMPOUNDS[1,2,3]


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The development of the technology of plant growth substances has resulted in the recognition of various chemicals which are useful in controlling various physiological processes in plants. One of the compounds which has been developed in this connection is maleic hydrazide[4] (1).

Several investigators have proposed suggestions to explain the action of maleic hydrazide on plants. Naylor and Davis (2) and Isenberg et al (3, 4) suggest that maleic hydrazide is a respiratory inhibitor, at least at higher concentrations. Greulach and coworkers (5, 6) report that maleic hydrazide restricts growth primarily by inhibiting mitosis. Leopold and Klein (7) suggest that it is an antiauxin. Other investigators (8, 9) believe that maleic hydrazide inhibits plant growth by preventing normal phloem differentiation and subsequent translocation of carbohydrates.

Only Muir and Hansch (10) have proposed a chemical explanation of a possible mechanism whereby maleic hydrazide inhibits growth. They suggest that maleic hydrazide acts as a growth inhibitor by reacting with the free physiologically essential thiol groups. This suggestion seemed likely in view of certain observations in the literature. First, a variety of substituted $\alpha$, $\beta$ unsaturated lactones have been observed to inhibit growth in plants (11, 12). Cavallito and Haskell (13) have further elucidated the mechanism of reaction between these lactones and cysteine, and have noted that free thiol group(s) do disappear with the formation of ring compounds. Second, Morgan and Friedman (14) noted that maleic acid, structurally related to maleic hydrazide, does react with a number of thiols. Hopkins and collaborators (15, 16) found that maleic acid reacted with the thiol groups of proteins and thereby inhibited succinic dehydrogenase activity. Although dehydrogenases in general do contain free thiol groups (17), some dehydrogenases investigated were not inhibited by the disappearance of free thiol groups. Maleic acid was effective in reducing succinic dehydrogenase action whereas other unsaturated acids, mesconic, citraconic, cis-cinnamic and trans-cinnamic, were without effect.

In order to learn if maleic hydrazide does inhibit growth by reacting with the free thiol groups, the interactions of maleic hydrazide with thiol compounds were investigated both in the presence and absence of plant tissue homogenates.

A technique similar to that used by Morgan and Friedman (14) was employed in this study. The influence of maleic hydrazide on the thiol was studied in two ways. First, the particular thiol compound and maleic hydrazide were prepared in a solution at a concentration of about 0.2 molar with respect to each substance (volume 50 ml) and at a pH of 7.0. Second, a tissue homogenate (10 gm) was used with maleic hydrazide and thiol compound at the same concentration and at the pH previously described. Radish leaf tissue which had been stored below 0° C was used as a source of biological material. Homogenates were prepared from this frozen material by macerating with two weight equivalents of water. A boiled homogenate was prepared by heating an aliquot of the homogenate in a boiling water bath for 10 minutes. The reaction mixtures, controls and reaction mixtures plus tissue homogenates were incubated up to 4 days at 37° C. To determine free thiol groups, 1.0 ml aliquots of the reaction mixture were taken at 0 time, and at definite time intervals after incubation. The amount of thiol in the aliquots was estimated by adding an excess of standard iodine solution and the excess iodine back titrated with standard thiosulfate using starch as the internal indicator. Blank solutions contained only maleic hydrazide, thiol compound, or tissue homogenate in quantities equivalent to those used in the reaction mixtures. Maleic acid was used in place of maleic hydrazide under similar conditions as a check on the experimental procedure.

The data confirm the observation that thiols will


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[2] Journal Article No. 1963 from the Michigan Experiment Station, East Lansing. This research was supported by the Horace H. Rackham Research Endowment of Michigan State University.


[3] From the Ph.D. thesis of Lowell E. Weller, Michigan State University, 1956.

[4] Maleic hydrazide is named systematically as 1,2-dihydropyridazine-3,6-dione.

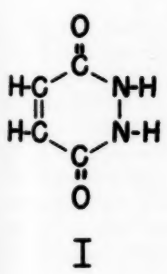
TABLE I

INTERACTION OF THIOL COMPOUNDS AND MALEIC HYDRAZIDE

| SYSTEM COMPONENTS | INCUBATION TIME (DAYS) | | | |
|---|---|---|---|---|
| | 0 | 1 | 2 | 4 |
| | *Meq × 10³ of iodine oxidized by the thiol compound in a 1.0-ml aliquot of solution* | | | |
| Maleic hydrazide (blank) .. | 0 | 5 | 2 | 3 |
| Mercaptoacetic acid (blank) | 185 | 184 | ... | 182 |
| Maleic acid and mercaptoacetic acid (check) ..... | 191 | 31 | ... | ... |
| Maleic hydrazide and mercaptoacetic acid ..... | 190 | 189 | ... | 187 |
| Maleic hydrazide and cysteine | 188 | 186 | ... | 183 |
| Maleic hydrazide and glutathione (G-SH) ..... | 189 | 186 | ... | 185 |
| Tisses homogenate ..... | 0 | 0 | 4 | 1 |
| Boiled tissue homogenate ... | 0 | 0 | 3 | 0 |
| Homogenate and mercaptoacetic acid ..... | 183 | 183 | 185 | 182 |
| Boiled homogenate and mercaptoacetic acid ..... | 185 | 183 | 187 | 185 |
| Homogenate, mercaptoacetic acid and maleic hydrazide | 186 | 186 | 187 | 185 |
| Boiled homogenate, mercaptoacetic acid and maleic hydrazide ..... | 188 | 186 | ... | 183 |

react with maleic acid to render them unavailable for titration (table I). However, the results also indicate that maleic hydrazide does not react with the thiol group under conditions similar to these used to ascertain the addition of maleic acids and thiols. Further studies using tissue homogenates indicate that under these conditions there is no evidence of an enzyme system in these tissues capable of catalyzing the reaction of thiol radical with maleic hydrazide or converting the maleic hydrazide into a compound that would add to the thiol group. These results further indicate that the hypothesis of Muir and Hansch (10), that maleic hydrazide inhibits growth by reacting with essential free thiol groups similar to addition of thiol compounds to the double bond of maleic acid, is not a very probable one.

If the structure of maleic hydrazide were as usually represented (structure I), the addition typical of an ethylenic double bond would be expected; for example, the addition of thiol to maleic acid as previously mentioned. However, the methylation studies of Arndt and co-workers (18, 19) and the recent study of the ultraviolet spectra of the methyl derivatives of maleic hydrazide by Miller and White (20) indicate that maleic hydrazide exists as structure II, i.e., 6-hydroxy-3(2H)pyridazinone. The results of this investigation indirectly substantiate structure II. The ring in structure II has become partially aromatized and as a result is not capable of the addition reaction in question.



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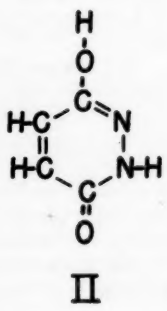

II

## SUMMARY

The observation that thiols will form addition compounds with maleic acid was confirmed. However, maleic hydrazide, which is structurally related to maleic acid, does not react with thiol groups under conditions similar to those used for the addition of maleic acid to thiols. Studies using radish leaf tissue homogenate indicate that under the conditions studied there is no evidence of an enzyme system in these tissues capable of catalyzing the reaction of thiol radical with maleic hydrazide or converting the maleic hydrazide into a compound that would add to the thiol group.

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# FOLIAR SYMPTOMS OF DEFICIENCIES OF INORGANIC ELEMENTS IN TOMATO [1]

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Keys to deficiency symptoms with various elements known to be essential to the growth of higher plants have been published (1, 2, 5, 6, 8). These have been of significant general service to physiologists. Some tabulations may have certain limitations for students of mineral nutrition when assembled from uncoordinated observations of numerous workers with diverse species under dissimilar cultural conditions. In studies of deficiency diseases in this laboratory the need for a critical survey of symptoms was recognized. The results of replicated series of experiments with a favorable test plant are reported.

## MATERIALS AND METHODS

Experiments were carried out with tomato plants (*Lycopersicum esculentum* L.) during relatively early stages of rapid vegetative development. Treatments with and without intentional supply of single elements were replicated, and tested at least two separate times, so as to characterize the symptom as typical of the expected deficiency.

Germination and general cultural conditions are published elsewhere (3). In outline, germination was made over washed cheese cloth suspended over a dilute macronutrient supply contained in shallow Pyrex or polyethylene vessels. Seedlings were transferred at an early first-true-leaf stage of development (about 10 days) to solution culture media contained in 4-l Pyrex or polyethylene receptacles. Polyethylene ware was used in the boron studies; Pyrex ware, for other elements. Solutions were continuously aerated and maintained at temperatures favorable for vigorous plant development. Cultures were maintained under usual greenhouse conditions with the ambient air supply largely filtered through carbon-limestone filters. The filters aided in restricting possible damage from smog, and extraneous supply of the test element from aerosol, gas or dust.

Macronutrient deficiency studies were made with a variable, commercial grade, salt supply combination appropriate to the element in question (3). A standard nutrient medium (solution 1 (3)) was used as the control. Initial supplies were augmented to maintain all but the test element at adequate levels, during study. Micronutrients, except Cl, were supplied at adequate levels from ethylenediamine tetra-acetate; chlorine was supplied from KCl solution.

Micronutrient deficiency studies were made with a uniform macronutrient supply (4). Micronutrients were added as salts (without ethylenediamine tetra-acetate) as described elsewhere (4), omitting that one concerned with the element under investigation. Here, all salts were doubly recrystallized prior to preparation of solutions. It has been observed, however, that pronounced deficiencies for all elements except molybdenum may obtain (at least at later stage of development) where current reagent grade salts are used.

The tomato plant was used throughout the study. This species was selected for several reasons. First, this plant has been used extensively in the past for mineral nutrition studies. Second, its morphology is such as to allow distinct separation of plant organs, for example, roots, stems and leaf petioles, leaf laminae, fruit, and seed. Third, the plant lends itself readily to radioautography, as a whole or in its parts. Fourth, under usual culture conditions, it is quite free of fungal and virus disease, and is fairly resistant to

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## TABLE I

### KEY TO NUTRIENT DEFICIENCY SYMPTOMS ON TOMATO PLANTS

| | |
|---|---|
| $A_1$ Symptoms appearing first or most severely on youngest leaves | B |
| $B_1$ Interveinal chlorosis present on young leaves | C |
| $C_1$ Black spots appear adjacent to veins. Smallest veins remain green. In older leaves the necrosis may appear as interveinal brown necrotic spots 2 to 4 mm in diameter located near the main veins | MANGANESE |
| $C_2$ Black spots do not appear. Smallest veins do not remain green. Necrotic areas, if present, are not associated with any particular part of the lamina | IRON |
| $B_2$ Young leaves do not show *interveinal* chlorosis, but young leaflets may show chlorosis toward the central basal portion of the leaflets | D |
| $D_1$ Dorsal sides of young leaflets show marked purpling. This purpling includes both veins and interveinal areas. The ventral surfaces of young leaflets are very dark green. Leaflets are small and curled downward. Oldest leaves may show slight interveinal chlorosis and necrosis | PHOSPHORUS |
| $D_2$ Dorsal sides of young leaflets are not purple. Ventral surfaces of young leaflets often show central basal chlorosis. Growth may be distorted. Interveinal necrosis often appears at bases of young leaflets. Very young growing tissue shows necrosis | E |
| $E_1$ Plant tissues are very brittle, especially under conditions of low stress for water. Growth accompanying recovery from this deficiency is usually twisted, asymmetrical, and otherwise distorted | BORON |
| $E_2$ Plant tissues are soft and often flaccid even under conditions of low stress for water. Leaflets developing after onset of the deficiency are narrow and cupped downward, but there is not usually much twisting either under deficiency conditions or upon recovery | CALCIUM |
| $A_2$ Symptoms neither appearing first nor being most severe on youngest leaves. Symptoms about equal over entire plant, or most severe on oldest or on recently matured leaves | F |
| $F_1$ Interveinal chlorosis present, possibly only as a mild mottling | G |
| $G_1$ Oldest leaves most chlorotic | H |
| $H_1$ Chlorosis definitely interveinal, so that at least the main veins remain green. Plants are not usually spindly | I |
| $I_1$ Chlorosis is the first visible symptom. Leaf edges curl upward in severe deficiencies | J |
| $J_1$ Necrosis appears as sunken necrotic spots which appear shiny from the back of the leaf. These spots have no particular location with respect to veins. Bright yellow and orange colors of the chlorotic leaves are common | MAGNESIUM |
| $J_2$ Necrosis occurs as gradual drying of interveinal areas followed by drying of the remaining tissues. Bright coloration is not common in chlorotic leaves | MOLYBDENUM |
| $I_2$ Chlorosis is not the first visible symptom. Leaf edges do not usually curl upward | K |
| $K_1$ Tip and marginal necrosis present on older leaves of mildly affected plants, appearing as a "scorch". Old or recently matured leaves may show interveinal chlorosis. Leaflets sometimes show small black interveinal necrotic dots. Neither excessive guttation nor "water soaking" is present | POTASSIUM |
| $K_2$ Tip and marginal necrosis is absent. Only the oldest leaves of severely damaged plants show chlorosis. Necrosis appears as irregular sunken necrotic spots which may be veinal, interveinal, or adjacent to veins. Young leaflets sometimes show small black interveinal necrotic spots. At times of low stress for water, excessive quttation and "water soaking" of the leaf tissues may be observed | ZINC |
| $H_2$ Chlorosis general, so that veins do not remain green. Plants are often spindly | L |
| $L_1$ Veins become bright red. Petioles and petiolules tend to be twisted and/or vertically disposed | SULPHUR |
| $L_2$ Veins are yellow or possibly somewhat pink. Petioles and petiolules do not show twisting or vertical disposition | NITROGEN |
| $G_2$ Oldest leaves not most chlorotic. Chlorosis, when present, appears as interveinal mottling on recently matured leaves. Small black necrotic dots appear on young or recently matured leaves | M |
| $M_1$ Tip and marginal necrosis is present on older leaves of mildly affected plants, appearing as a "scorch". Old or recently matured leaves may show interveinal chlorosis. Leaflets sometimes show small black interveinal necrotic dots | POTASSIUM |
| $M_2$ Neither tip nor marginal necrosis is present on older leaves until the entire plant is severely affected. Some tip necrosis is often present on the youngest leaves. Younger leaflets often show small black interveinal necrotic dots, especially adjacent to the main veins. Necrosis is usually confined to interveinal tissues adjacent to main veins | MANGANESE |

TABLE I (*Continued*)

| | |
|---|---|
| $F_2$ Interveinal chlorosis is not present | N |
| $N_1$ Leaf margins and tips wilt. Leaves do not show excessive guttation | O |
| $O_1$ Necrotic spots, when present, are sharply delimited and sunken. Old leaflet margins roll upward stiffly. Veinal necrosis is often present. Petioles and petiolules often bend abruptly and stiffly downward | COPPER |
| $O_2$ Necrosis appears as bronze-colored areas of cellular necrosis. These areas are neither sunken nor sharply delimited. *Old* leaflet margins are not usually rolled upward. Veinal necrosis is not present. Petioles and petiolules do not show abrupt downward bending | CHLORINE |
| $N_2$ Leaves show no excessive wilting except in cases of petiole necrosis. Wilting, if present, is not confined to leaflet tips and margins. Excessive guttation occurs under conditions of low stress for water. This is often accompanied by the appearance of water-soaked areas on the backs of the leaves. Necrosis usually appears as irregular spots which may or may not have any particular relationship to the veins | ZINC |

smog and fumigants. Fifth, foliar symptoms appear marked and distinct. Sixth, it is a vigorous, erect grower under conditions in this and probably most areas when under glass. Seventh, tomato seed is small and supply of a test element per seed is usually little, except where seed is possibly derived from plants previously supplied with excessively high levels of the element in question.

Five plants were transplanted per culture; thinned to the four most uniform at about one week, leaving one plant per liter of nutrient medium. Generally, deficiency symptoms were observed with plants untreated (apart from intrinsic contamination of salts and water) with respect to a single essential element. Time of effect varied with season, usually being earlier in summer. Often deficiency symptoms (macronutrient especially) were observed early and drastically. To observe effects on more mature plants, some were treated early with an amount of test element inadequate to supply the plant for a prolonged time. Symptoms at younger stages were compared with those appearing at times later in the ontogeny of the plant.

## OBSERVATIONS AND RESULTS

A key to foliar deficiency symtoms is presented as table I. Possible symptoms were photographed and cross checked with ones observed in duplicate experiments to establish with reasonable certainty that the effect was typical of the expected disease. Interactions between elements (e.g., copper-induced iron chlorosis) were not studied. Color transparencies have been catalogued and filed for each of the typical symptoms. Many of these, with description, are available through the National Plant Food Institute (7).

## DISCUSSION

Deficiency of inorganic elements for growth and development of plants is determined by the adequacy of supply to the organism. This implies a continuing availability from the plant environment, an adequate rate of absorption and translocation, and possibly remobilization. The ability to observe foliar symptoms, and their manifestation, may be altered by environmental factors and is determined by the rates of internal physico-chemical conditions and processes.

There are numerous factors which may modify the degree of apparent deficiency symptoms. Some of these are season, stage of ontogeny at which deficiency sets in, external environmental circumstances, interactions of ions, and inadvertent external contamination. With controlled solution cultures, disease is rather clear cut. With soils, if a single deficiency is concerned, symptoms should be likewise definite. However, with the inherent complexity of plant-soil interrelations it often may become more difficult to diagnose a malnutrition; though a key is of use, experience in characterization is also helpful. This key has been developed from careful observations with tomato; relative position in the tabulation may shift with species, especially for those types with different habit.

## SUMMARY

A key to foliar symptoms of mineral deficiencies with tomato is presented. This is a distinct aid in characterizing malnutrition conditions under controlled solution culture as well as in the field. Early recognition of such situations, augmented possibly by foliar analyses, should be of service in agricultural practice.

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# GROWTH- AND ENZYME-INHIBITING PROPERTIES OF BEAN SEED EXTRACTS[1]

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Integuments and other parts of the Red Kidney bean seed (*Phaseolus vulgaris* var. Red Kidney) have been demonstrated to contain germination and growth inhibitors (1, 2, 3), but seed extracts have not previously been characterized further save for limited data on their solubility and absorption spectra. In the course of an investigation of peroxidases, an opportunity to characterize further these seed coat extracts was obtained.

Seed coat preparations were made by extracting washed seed coat fragments 1 mm or less in diameter removed from dry seeds in water at 100° C for 10 minutes, 1 gm of seed coat/20 ml water, followed by filtration to produce a clear to slightly turbid orange-brown solution. This solution contained 5 to 10 mg of dissolved matter per ml, the dry solids consisting of 15 to 20 % ether-solubles; 22 to 27 % ethanol-solubles (ether-insoluble); and 63 to 53 % water-solubles (insoluble in ether and ethanol). Water- and ethanol-soluble fractions gave blue-gray or gray colors with $FeCl_3$ as do gallic acids and tannins; a yellow color in nitrating mixtures; and yellow-green color in $NH_3$ vapor. The aqueous fraction reduced Fehlings solution directly, but reduction was more extensive after treatment with $\beta$-glucosidase. The ethanol fraction, when redissolved in water, showed no additional reducing activity after enzyme treatment. The ether fraction gave no $FeCl_3$ test, but was yellowed by $NH_3$ or dilute NaOH. Ultraviolet absorption spectra, determined with the Beckman model DU spectrophotometer, were typical for phenolic compounds, with well-defined maxima in the 260 to 280 m$\mu$ region, and with E (liter/gm × cm) values of 10 to 17. Preliminary chromatographic separation of the original solution (Whatman No. 1 paper, butanol-6 *N* ammonia 1 : 1 v/v) gave six spots, over the range $R_f$ 0 to 0.95 yielding positive tests with phenol reagents. On the basis of these data, it may be assumed that the inhibitory activities reside in one or more phenolic compounds present in the extracts.

Peroxidase activity was measured photometrically by the rate of formation of purpurogallin (max 425 m$\mu$) from pyrogallol in 10-ml reaction mixtures containing $10^{-3}$ M pyrogallol, $5 \times 10^{-3}$ M $H_2O_2$; $10^{-2}$ M $KH_2PO_4$, 1 ml of a suitable peroxidase preparation and varying amounts of unfractionated seed coat extract. Peroxidase sources include particle-free breis of bean-embryo, pea root, and Elodea internode, as well as raw fat-free milk.

For growth studies, an experiment consisted of duplicate sets of 25 flax seeds (var. Bison, 1955 commercial crop) each germinated in Petri dishes for 3 days in darkness at 26° C with varying amounts of unfractionated aqueous extract added. Two such experiments were carried out in toto. Peroxidase and phenolase assays on flax root breis were carried out using the peroxidase technique described above, save that the phenolase was measured without peroxide and the former was taken as the difference in rate of pyrogallol oxidation with and without peroxide. Nitrogen analyses were done on trichloroacetic acid soluble and precipitable fractions of root breis by the usual sulfuric acid digestion, Nesslerization and direct photometric estimation of N as $NH_3$.

All of the peroxidases tested were inhibited by

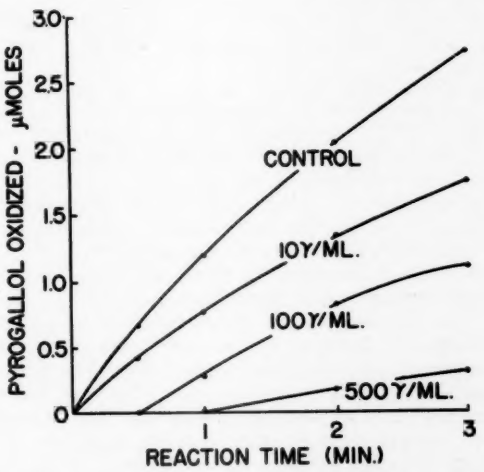


FIG. 1. Time course of pyrogallol oxidation by $H_2O_2$ in the presence of Elodea peroxidase with varying amounts of aqueous seed coat extract added. Assays run at 25° C, pH 4.5, with $10^{-3}$ M pyrogallol, $5 \times 10^{-3}$ M $H_2O_2$, and $10^{-2}$ M buffer. Each rate curve is based on an average of 3 determinations with a maximum variation among replicates of 1.5 %.

[1] Received December 1, 1956.

TABLE I

EFFECT OF SEED COAT EXTRACTS ON GROWTH COMPOSITION OF 3-DAY-OLD SEEDLINGS *

| CHARACTERISTIC | INHIBITOR (MG/PETRI DISH) | | |
|---|---|---|---|
| | 0 | 2.5 | 7.5 |
| Germination (%) | 80.0 | 64.0 ** | 32.0 ** |
| Root length (mm) | 11.6 | 8.6 ** | 6.1 ** |
| Root fresh wt (mg) | 7.2 | 6.3 | 3.7 ** |
| Protein N (μgm/root) | 44.9 | 31.1 ** | 28.0 ** |
| Soluble N (μgm/root) | 9.9 | 11.5 | 10.5 |
| Peroxidase (mμmoles pyrogallol oxidized/min per root) | 62.4 | 63.0 | 38.4 ** |
| Phenolase (mμmoles pyrogallol oxidized/min per root) | 25.2 | 23.9 | 20.7 ** |
| Root color | yel.-white | lt. brwn. | dk. brwn. |

* Germination based on 4 replicates, 25 seeds each; growth and analytical data based on 4 replicates of 15 to 20 roots each, save for the most inhibited group which provided duplicate samples of 16 roots.

** Significance. P = 0.05 or less (difference between control and treated).

seed coat extracts to about the same degree. Data for inhibition of Elodea peroxidase (fig 1) are representative, and show both a suppressed rate of oxidation and delay in the start of oxidation, the latter suggesting an anti-oxidant effect.

Data on growth and composition of the flax seedling (table I) show suppression by seed coat inhibitors both of germination and root growth; reduction in protein; and reduction in the total peroxidase and phenolase activity per root. Discoloration of root tissue was common with the lower level of inhibitor and extreme in all roots given the higher level. It has also been noted that incubation of bean embryo and flax root slices in seed coat extract–$H_2O_2$ mixtures results in deposition of brown pigments in the slices implying at least that peroxidase can utilize seed coat phenols as substrates.

## SUMMARY

The work described above does not necessarily point to the mechanism whereby seed coat components act as growth inhibitors, however, it is possible that seed coat phenols either interfere directly with protein synthesis or are first converted into highly toxic quinonoid substances by endogenous tissue peroxidase.

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# PHOTOSYNTHESIS IN GIBBERELLIN-TREATED LEAVES [1,2]

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A major effect of gibberellins on plants has been reported (2, 3) as producing increased rates of growth in size and weight. Such growth could be the result of either an increased rate of photosynthesis, or a more efficient or altered utilization of the photosynthetic products. To explore these possibilities the rate of $C^{14}O_2$ fixation and the distribution of $C^{14}$ among the products from short-time photosynthesis in gibberellin-treated plants has been studied

Basal ends of single detached leaves from 2- to 3-week-old plants of Sacramento barley, *Hordeum vulgare* L. (4 experiments), Fork Deer oats, *Avena sativa* L. (2 experiments) and Alaska peas, *Pisum sativum* L. (3 experiments) were set in vials containing solutions of gibberellic acid (Merck & Co., No. 56R3543) ranging from zero to 1000 ppm. The leaves took up the solutions in the transpirational stream for 2 hours while standing in aerated photosynthesis chambers that were immersed in a water bath at 21° C and exposed to about 1000 ft-c of white light. The chambers were then sealed, 12.5 μc $C^{14}O_2$ released inside, and the leaves allowed to photosynthesize for 30 minutes longer. They were then killed in boiling 85 % alcohol and further extracted with hot and cold water. Aliquots of the combined extracts were counted for total fixed $C^{14}$. Other aliquots were chromatographed for examination of the products of

TABLE I

FIXATION OF $C^{14}O_2$ BY OAT LEAVES 2 HOURS AFTER BEGINNING GIBBERELLIC ACID TREATMENT

| CONC. OF GIBBERELLIC ACID | TOTAL $C^{14}O_2$ FIXED PER LEAF * |
|---|---|
| *ppm* | *cts/sec* |
| 0 | 24,300 |
| 0.1 | 23,300 |
| 1 | 21,000 |
| 10 | 25,600 |
| 100 | 24,600 |
| 1000 | 21,000 |

* Total $C^{14}O_2$ fixed in 30 min into hot alcohol and water soluble constituents by the apical 4.5 inches of a leaf.

[1] Received December 31, 1956.
[2] Work performed under Contract No. W-7405-eng-26 for the U. S. Atomic Energy Commission.

TABLE II

FIXATION OF $C^{14}O_2$ BY PEA LEAVES AFTER GIBBERELLIC ACID TREATMENT *

| CONC. OF GIBBERELLIC ACID | TOTAL $C^{14}O_2$ FIXED/MG LEAF DAYS AFTER SPRAYING | | | AVERAGE HT DAYS AFTER SPRAYING | | |
|---|---|---|---|---|---|---|
| | 3 | 6 | 9 | 3 | 6 | 9 |
| *ppm* | *cts/sec* | | | *cm* | | |
| 0 | 208 | 171 | 124 | 10.0 | 13.0 | 19.0 |
| 1 | 183 | 141 | 137 | 13.5 | 14.0 | 21.0 |
| 10 | 186 | 151 | 99 | 16.0 | 17.5 | 25.0 |
| 100 | 179 | 201 | 136 | 16.0 | 23.5 | 26.0 |

* Experiments at 3 and 6 days after spraying were run with one group of plants and at 9 days with a different group.

photosynthesis (1). Total radioactivity fixed per leaf was unchanged by the two-hour treatment with gibberellic acid; typical results are shown in table I. Similar results were also obtained in one experiment with *Chlorella pyrenoidosa*. Examination of autoradiograms of the chromatograms of extracts from the oats, barley, and peas showed no significant differences in the distribution of $C^{14}$ among the soluble products within any one species.

Experiments were also performed 2 days after one-week-old oats, and 3, 6, and 9 days after 9-day-old peas had been sprayed with gibberellic acid. In these experiments the leaves were put in vials containing water in the photosynthesis chamber for 15 minutes and then immediately exposed to $C^{14}O_2$ for 20 minutes. There was no significant difference in amount of $C^{14}O_2$ fixation or in distribution of $C^{14}$ among the products of photosynthesis even though the plants had shown a typical response to gibberellic acid (table II).

## SUMMARY

Both types of experiments indicate that gibberellic acid did not enhance the rate of $CO_2$ fixation per unit of leaf tissue and did not alter the general pathways of short-time metabolism of the newly fixed $C^{14}O_2$ in the sugars, organic acids, and amino acid products.

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# A PAPER CHROMATOGRAPHIC METHOD FOR THE DETECTION OF 3-AMINO-1,2,4-TRIAZOLE IN PLANT TISSUES [1]

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During the course of investigation of the physiological mode of action of 3-amino-1,2,4-triazole, otherwise called aminotriazole, ATA or "Amizol," it was necessary to develop a technique for identifying minute amounts of this compound in extracts of plant tissues. It was found that aminotriazole would produce, after diazotization, a characteristic yellow color when coupled to phenol. The reaction goes rapidly on filter paper and appears to be specific for 3-amino-1,2,4-triazole. The reaction is illustrated in figure 1. Histidine, adenine and guanine, which have structural resemblance to aminotriazole, gave no color when solutions of them were spotted on filter paper and treated similarly.

Extracts of Black Valentine bean and Baart wheat leaves were prepared by grinding fresh tissue in a mortar with a small amount of washed sand. To the slurry was added sufficient ethanol to maintain an 80 % alcohol concentration in the homogenate. Grinding was continued until all fibers were reduced to a uniform pulp. The homogenized tissue, plus sand, was then transferred to a Büchner funnel and the solvent removed over vacuum. The residue was washed several times with small aliquots of 80 % ethanol (v/v) until no pigment was visible in the eluate. Each extract sample was reduced in volume to 5 or 10 ml under vacuum at room temperature. All samples were brought to an identical volume if quantitative estimations were to be made of the aminotriazole present in the extracts.

An ascending sheet or strip method, using Whatman No. 1 filter paper, was used for chromatographic separation of aminotriazole from other components present in the extracts. Samples were spotted on the paper by means of a micropipette. Ethanol : water : 1-butanol, 1 : 1 : 4 (v/v) was employed as a solvent system for separating the extract components. One or two drops of pyridine added to each liter of the solvent mixture improved resolution of the yellow aminotriazole spots, especially when equilibration of the chamber was not complete before development of the chromatograms began. The chromatograms were run at room temperature (20 to 25° C) for sufficient

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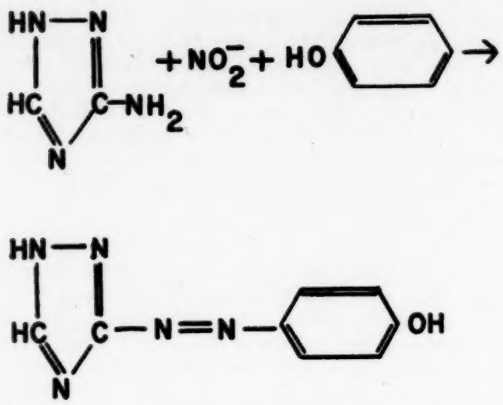


FIG. 1. Formation of a yellow azo dye by coupling diazotized 3-amino-1,2,4-triazole to phenol.

time to allow the solvent front to rise at least 10 cm. After development, the paper was removed from the chamber and air-dried.

Reagents used for producing the aminotriazole yellow reaction are: 1) A 5 % (w/v) solution of purified $KNO_2$ in distilled water. The reagent should be as free from nitrate as possible. Some commercial samples required recrystallization in order to prevent browning of the filter paper. 2) A saturated solution of chromatographic-grade phenol in 20 % (v/v) HCl. Both solutions should be made up fresh before each use.

The chromatograms were first sprayed with the $KNO_2$ solution, using a glass or plastic atomizer. Before the paper dried, the phenol-HCl reagent was similarly applied. Spots containing aminotriazole appeared rapidly as lemon-yellow areas of clear definition. The minimum detectable amount of aminotriazole was found to be ca 0.1 microgram per spot.

This method has also been applied with success to the detection of aminotriazole in cow's milk. It may be noted that two yellow spots appear on chromatograms of plant extract or milk. This is presumed to be caused by the presence of "free" and "bound" aminotriazole in the preparations. Aminotriazole appears to bind tightly to proteins. Free aminotriazole appears at $R_f$ 0.45 to 0.48 and the bound fraction moves slowly, if at all, appearing at $R_f$ 0.025 to 0.038. The bound aminotriazole did not dissociate upon addition of acid or base to the extracts prior to chromatographing. Both spots are attributable to aminotriazole since each exhibits an absorption maximum of 460 m$\mu$, as does a test-tube preparation of the aminotriazole azo dye.

"Amizol" is the registered trade mark of the American Chemical Paint Co., Ambler, Pennsylvania, for 3-amino-1,2,4-triazole. This concern supplied purified samples of aminotriazole.

American Society of Plant Physiologists, *Appointments:* Harry A. Borthwick has been appointed as our representative to the AIBS Committee on Travel Grants to the International Cell Biology Congress to be held in August in St. Andrews, Scotland.

Donald B. Anderson has been appointed our representative on the National Register of Scientific Personnel.

Lawrence Blinks has been appointed our local representative at the Stanford campus for the AIBS meetings in August—Arthur W. Galston, *Secretary.*

## HENRY REIST KRAYBILL

The American Society of Plant Physiologists has lost one of its former officials and an outstanding long time member by the recent death of H. R. Kraybill. While listing himself as an *agricultural chemist* or as a *biochemist,* Dr. Kraybill was quite active in plant physiology and gained professional recognition as a *plant physiologist.* This is testified by his election as Secretary-Treasurer (1928–1930) and President (1931) of our Society. His earlier research and publications dealt primarily with plants, of which the most important seem to be the biochemical diagnosis of "false blossom" of cranberry as a virus disease, the nitrogen composition of re-sweated tobacco leaves and the relation of carbohydrate and nitrogen metabolism to growth and reproduction of the tomato. The very extensive chemical research on the tomato, conducted cooperatively with Dr. E. J. Kraus and published as a joint Ph.D. thesis from the University of Chicago (Oregon Agricultural Experiment Station Bulletin 149, 1918) undoubtedly gained the greatest reputation for Dr. Kraybill as a plant physiologist. During the last 15 years of his life (1941–1956) Dr. Kraybill was associated with the American Meat Institute in Chicago and his interests were quite exclusively with biochemistry of animal tissues. He became well known for his work on meat processing, oils and fats, antioxidants, phosphatides, sterols and the effects of processing on the nutritive value of meats.

Dr. Kraybill's personal qualifications, an unusually pleasant personality and administrative ability, were recognized early in his career and put to good use. After receiving the B.S. degree in Agricultural Chemistry from the Pennsylvania State College in 1913, advanced degrees from the University of Chicago, and a brief service with the U. S. Department of Agriculture, he was appointed Professor of Agricultural Chemistry and Head of the Department of University of New Hampshire (1919–1924). This was followed, with a brief interlude as biochemist at the Boyce Thompson Institute for Plant Research, by appointment as Professor and Head of Department of Agricultural Chemistry at Purdue University, where he performed creditable service for 15 years (1926–1941). In 1941 Dr. Kraybill joined the American Meat Institute as Director of Scientific Research and simultaneously was appointed a lecturer in biochemistry at the University of Chicago. From 1947 till his death he was also Director of Research and Education of the American Meat Institute Foundation, thus holding three professional appointments.

Because of his outstanding services, many honors were bestowed on Dr. Kraybill. In addition to being recognized as a leader by the American Society of Plant Physiologists, he was a former president of the Association of Agricultural Chemists (1938) and the American Association of Feed Control Officials. He served also as chairman of the Division of Agricultural and Food Chemistry of the American Chemical Society (1936–1938) and was a Scientific Advisor to the Frasch Foundation for Research in Agricultural Chemistry (1939–1947). In June, 1956, Purdue University conferred on him the honorary degree of Doctor of Science "in recognition for his distinguished achievements as a scientist, teacher and administrator, whose contributions in biochemistry, plant production and food processing have advanced the science of agriculture, resulting in benefits to all people." Just prior to his death Dr. Kraybill was notified that he had been designated as the recipient of the 1956 Dodge and Olcott Award for his scientific accomplishments of outstanding and lasting significance in connection with processing of meat and meat products, specifically the recognition and development by him and his associates of two food antioxidants of great practical value. Thus his death occurred at the apparent height of public recognition of his professional accomplishments.

Dr. Kraybill died in Chicago on September 30, 1956.—A. E. Murneek.

The Chemistry and Mode of Action of Plant Growth Substances—R. L. Wain and F. Wightman, eds. Academic Press, Inc., New York. 312 pages. 1956. $9.50.

The papers presented in this volume were read at a conference on plant growth substances at Wye College, University of London, by a number of active investigators in this field. The contributions are arranged in chapters discussing: 1) natural auxins, 2) chemical structure and biological activity, 3) metabolism and mode of action, and 4) application of kinetics to auxin-induced growth.

Natural Auxins: Methods for the investigation of natural auxins and growth inhibitors, J. P. Nitsch; The distribution of natural hormones in germinating seeds and seedling plants, Phyllis M. Cartwright, J. T. Sykes and R. L. Wain; Hormones and hormone precursors in leaves, roots and seeds, Foyce A. Bentley, S. Housley and G. Britton; Chromatographische Untersuchungen über die Wuchsstoffe und Hemmstoffe der Haferkoleoptile, H. Söding und Edith Raadts; Indole compounds in photoinduced plants, A. J. Vlitos; The biogenesis of natural auxins, S. A. Gordon; Geotropic responses in roots. Some theoretical and technical problems, P. Larsen.

CHEMICAL STRUCTURE AND BIOLOGICAL ACTIVITY: On the effects of para-substitution in some plant growth regulators with phenyl nuclei, B. ABERG; On form and function of plant growth substances, H. VELDSTRA; The mode of growth action of some naphthoxy compounds, H. BURSTRÖM and BERIT A. M. HANSEN; Chemical configuration and action of different growth substances and growth inhibitors: New experiments with the paste method, H. LINSER; The action-concentration curves of mixtures of growth-promoting and growth-inhibiting substances, K. KAINDL; The chemical induction of growth in plant tissue cultures, F. C. STEWARD and E. M. SHANTZ; The degradation of certain phenoxy acids, amides, and nitriles within plant tissues, C. H. FAWCETT, H. F. TAYLOR, R. L. WAIN and F. WIGHTMAN; Relationship of molecular structure of some naphthyloxy compounds and their biological activity as plant growth regulating substances, L. C. LUCKWILL and D. WOODCOCK; Studies on the relation between molecular structure and penetration of growth regulators into plants, J. VAN OVERBEEK; The resolution of phyto-hormone acids by means of their optically active phenylisopropylamides. Resolution of $\alpha$-(1-naphthyl)-propionic acid, J. M. F. LEAPER and J. R. BISHOP.

METABOLISM AND MODE OF ACTION: Some metabolic consequences of the administration of indoleacetic acid to plant cells, A. W. GALSTON; Chromatographic investigations on the metabolism of certain indole derivatives in plant tissues, R. C. SEELEY, C. H. FAWCETT, R. L. WAIN and F. WIGHTMAN; The effects of synthetic growth substances in the level of endogenous auxins in plants, L. J. AUDUS and RUTH THRESH; Interrelationships between the uptake of 2,4-dichlorophenoxyacetic acid, growth and ion absorption, G. E. BLACKMAN; Auxin-induced water uptake, J. BONNER, L. ORDIN and R. CLELAND; The influence of growth substances upon sulfhydryl compounds, A. C. LEOPOLD and C. A. PRICE; Salt accumulation and mode of action of auxin. A preliminary hypothesis, T. A. BENNET-CLARK.

APPLICATIONS OF KINETICS TO AUXIN-INDUCED GROWTH: The kinetics of auxin-induced growth, J. BONNER and R. J. FOSTER; The kinetics of auxin-induced growth, T. A. BENNET-CLARK.

During the last 25 years, an impressive number of publications dealing with problems of plant growth substances have appeared. This increase in interest has largely been due to the important role which plant hormones and their analogues have played in many phases of agricultural practices. It is, therefore, not surprising that investigations dealing with easily available indoleacetic acid and its derivatives received most attention, and this symposium was a typical example of this trend in plant physiology.

Its title: "The Chemistry and Mode of Action of Plant Growth Substances" could more properly have been, "Physiological and Chemical Studies on Indoleacetic Acid and Related Substances." Only two of the twenty-six papers would lead students in this field to suspect that there are probably a large number of plant growth substances completely unrelated to indoleacetic acid.

Nitsch, in his chapter on methods for the investigation of natural auxins and growth inhibitors, quite appropriately makes a plea for a really comprehensive survey of the native growth promoting and inhibiting substances in plants. His experimental work is, however, mostly concerned again with indole growth substances.

The papers presented in the symposium are good evidence of the difficulties in studying the physiology of the growth hormones. The occurrence of the hormones in extremely small quantities makes necessary special techniques, which are the subject of several contributions by different authors.

Experience has taught that theoretical conclusions from extraction procedures have to be accepted with caution. The ubiquitous presence of tryptophane, free and bound in proteins and the occurrence of tryptophane-oxidizing enzymes tends to give rise to trace quantities of indoleacetic acid, especially when tissues are macerated and many substances, which in the intact plants are kept apart, are released and can react.

Although two papers give suggestions regarding the mechanism of auxin actions, we can safely say that twenty-five years after the isolation of the auxins, we still have not found the solution to this problem. This is also true for other fundamental problems of plant hormone action—their transport and formation. In a field where there are still so many unknowns, a periodic appraisal of our knowledge is very useful.

On the whole, the collected papers give the reader a good insight into the present ways of thinking in the field of plant growth substances, and should be stimulating to further investigations.—A. J. HAAGEN-SMIT, *Division of Biology, California Institute of Technology, Pasadena, California.*